External quality assessment in flow cytometry

Educational aspects and trends toward improvement

Wilfried Levering

Colophon

Cover: This is a photograph of the flow cell of the FACScan™ flow cytometer (BD Biosciences) with serial number 83125. In this flow cell, one of the first data collections for the flow cytometric CD34+ stem cell enumeration EQA in the Benelux countries was performed. Camera: Nikon D100 with 105mm f/2.8D AF Micro-Nikkor, kindly provided by Tim Janse.

External quality assessment in flow cytometry
Improvements on qualitative and quantitative cell analysis

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External Quality Assessment in Flow Cytometry
Educational aspects and trends toward improvement

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Educatieve aspecten en trends tot verbetering

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Wilfridus Henricus Bernardus Maria Levering

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Promotor:	Prof.dr. D.J. van Rhenen
Overige leden:	Prof.dr. J.J. Cornelissen
	Prof.dr. W. Weimar
	Prof.dr. J. Philippé
Copromotor:	Dr. J.W. Gratama

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1

GENERAL INTRODUCTION

1.1 FLOW CYTOMETRY IN CLINICAL DIAGNOSTIC LABORATORIES

Flow cytometry (FCM) uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells. Cells are hydro-dynamically focused in a sheath of fluid before intercepting a focused (laser)light source. When cells of interest intercept the light source they scatter light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes. Scattered and emitted lights are converted to electrical pulses by optical detectors. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter (FSC)) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescence (FL) detectors). By analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak) it is then possible to extrapolate various types of information about the structure and other properties of each individual cell. For example, FSC correlates with the cell volume and SSC depends on the inner complexity of the cell (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Reagents like monoclonal antibodies (mAb), labeled with various fluorochromes, afford any desirable additional information for multi-parametric analysis of cells.

Nowadays, FCM has progressed from a research technique to a technology, which is indispensable for the routine clinical diagnostic laboratory. For example, FCM was introduced in the early 1980's to the clinical diagnostic laboratory for the enumeration of CD4+ T cells in patients infected with the human immunodeficiency virus (HIV). The introduction of the cluster of differentiation (CD) nomenclature in 1980 (1) greatly facilitated the communication of flow cytometric typing results, and consequently flow cytometry rapidly became the method of choice for lymphocyte immunophenotyping. In the 1990's, methods for flow cytometric detection of CD34+ hematopoietic stem and progenitor cells (HPC) in blood and apheresis products were published (reviewed in 2) which enabled the planning of apheresis procedures and the quantification of the engraftment potential of apheresis products for autologous and allogeneic stem cell transplantation (SCT). Nowadays, flow cytometric CD34+ cell enumeration gradually replaced the culture-based assays to quantify HPC in peripheral blood and bone marrow samples. In the same period, flow cytometry was introduced as a tool for HLA-B27 screening, which was used as a diagnostic criterion for e.g. ankylosing spondylitis (AS) (3). With the increased dependence on flow cytometry for clinical decision-making, various efforts to standardize testing and reduce variability between laboratories have been undertaken by many governmental and professional bodies. These efforts re-

sulted in the formulation of national and international guidelines. Such guidelines currently exist for CD4+ T-cell enumeration in HIV-infected patients (4-7), lymphocyte immunophenotyping (8-12), CD34+ cell enumeration (13-16) and screening for the HLA-B27 antigen (17,18). Clinical cell analysis by flow cytometry has entered the same process of development of instrumental validation, internal and external quality control (QC), and External Quality Assessment (EQA) as any other laboratory medicine activities. The need to guide medical treatment with reliable CD4+ T-cell counts, CD34+ stem cell counts, and HLA-B27 screening have been a stimulus to direct FCM as research technique towards the same accuracy and reproducibility as has been established for routine blood testing in clinical laboratories. The evolution of clinical cell analysis by flow cytometry is a good example of how the advent of a new technology can take advantage from the established laboratory quality issues.

In this thesis, we analyze the impact of these standardization efforts on clinical cell analysis by flow cytometry with special attention to the external quality assurance programs which currently are in operation for HLA-B27 screening, CD34+ cell enumeration, CD4+ T-cell enumeration, and lymphocyte immunophenotyping.

1.2 HLA-B27 SCREENING

1.2.1 Clinical background

Classical class I molecules, encoded in the major histocompatibility complex (MHC) at HLA-A, -B and -C loci, are extremely polymorphic with over 850 alleles identified (19). The majority of this variation occurs in HLA-A and -B, whose gene products are also expressed at much higher levels than HLA-C. MHC class I molecules consist of a heavy chain non-covalently bound to a short (8-11 residues) peptide and □2-microglobuline, as a result of a multifaceted process that occurs largely within the endoplasmic reticulum (reviewed in 20,21). The physiological function of MHC class I molecules is to display peptides on the cell surface for recognition by T-cell receptors on CD8+ T cells. The demonstration of the strong association between HLA-B27 and AS, a chronic inflammatory disease affecting the axial musculoskeletal system, has been recognized since 1973 (22,23). This association between HLA and disease, which is one of the few that are close to 100%, has been confirmed in many studies and extended to all major ethnic groups (reviewed in 3). In fact, HLA-B27 is common to the entire group of seronegative spondyloarthropathies, which also include Reiter's disease, subgroups of intestinal and psoriatic arthropathies, subgroups of juvenile rheumatoid arthritis and acute anterior uveitis (24). Furthermore, the heart has been identified as an important target for HLA-B27-associated disease. Specifically, atrioventricular conduction blocks and isolated aortic regurgitation are strongly associated with HLA-B27 (25). HLA-B27

designates a family of HLA-B locus alleles referred to as subtypes, 26 as assigned by the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System (B*2701 to B*2727) (19). The most common alleles are B*2702 (in Belgium, an allele frequency of 0.005 (26)) and B*2705 (in Belgium, an allele frequency of 0.071 (26)) which are both associated with AS (27). Consequently, screening for HLA-B27 is used for the differential diagnosis of patients with lower back pain and is, therefore, performed in many clinical diagnostic laboratories.

1.2.2 Technical aspects

A variety of methods has been developed to type for HLA-B27. The most common methods are the complement dependent cytotoxicity (CDC) assay (28), FCM (29), molecular typing based on DNA amplification like the polymerase chain reaction (PCR) with sequence specific primers (PCR-SSP) (30,31), PCR with sequence specific oligonucleotides (PCR-SSO) (32,33), and enzyme-linked immunosorbent assays (34). The classical assay for class I HLA typing is the CDC assay. The polyclonal typing reagents for and interpretation of this assay require the experience of a laboratory specialized in tissue typing. The advent and use of mAb specific for HLA-B27 enabled screening by flow cytometry for the HLA-B27 antigen by less specialized laboratories. However, flow cytometric HLA-B27 screening is complicated by the fact that HLA-B27 is member of a large cross-reacting group (CREG). CREGs consist of HLA antigens that share common epitopes (35). HLA-B27 is part of the HLA-B7 CREG that also includes HLA-B7, B13, B22, B40, B41, B42, B47 and B48. Cross-reactivity of HLA typing reagents, in particular human sera, is commonly encountered. Several mAb have been raised against

Table 1: Cross-reactivities of anti HLA-B27 mAb: summary of published studies

Cross-reactivity with HLA-B antigens[1]	Anti HLA-B27 mAb		
	ABC-m3	GS145.2	FD705
B7	27,144-150[2]	35,144,151	144
B12	27,144	144	144
B13	149	n.d.	n.d.
B17	n.d.	n.d.	144
B22	27,148,149	35,144	n.d.
B37	27,144	35,144	144
B39(16)	n.d.	35	n.d.
B41	n.d.	35	n.d.
B42	27,145	n.d.	n.d.
B73	27,145	n.d.	n.d.
none	n.d.	152	38,146

Table 1: [1] False-positive reactivity of HLA-B27 mAb with other HLA-B antigens
[2] Numbers refer to referenced publications
n.d. = not demonstrated

HLA-B27, such as FD705 (One Lambda, Canoga Park, CA), GS145.2 (Becton Dickinson Biosciences [BDBiosciences], San Jose, CA), and ABC-m3 (available through Behring [Brussels, Belgium], Serotec [Oxford, UK], Silenus Laboratories [Hawthorn, Australia] and Immunotech [Marseille, France]) (36-38). Unfortunately, HLA-B27-specific mAb cross-react too, usually with antigens within the HLA-B7 CREG (summarized in Table 1).

In this thesis we further analyzed the cross-reactivity patterns of commercially available anti-HLA-B27 monoclonal antibodies with an extensive cell panel with other HLA-B antigens in order to compose guidelines toward improvement of flow cytometric HLA-B27 screening (Chapter 3).

In an attempt to control for such cross-reactivities, the American Society for Histocompatibility and Immunogenetics (ASHI) and the European Federation for Immunogenetics (EFI) have included the following recommendation in their standards, complied by tissue typing laboratories, for serological HLA Class I typing in general as well as flow cytometric HLA-B27 typing (17,18).

It is known that the reactivity of anti-HLA-B27 mAb with the different HLA-B27 alleles (as defined by DNA typing) may vary. Currently, 27 HLA-B27 alleles are known and have been assigned by the WHO Nomenclature Committee for Factors of the HLA System (19). An overview of published reactivities with HLA-B27 alleles is shown in Table 2.

Table 2: Reactivity of various HLA-B27 monoclonal antibodies with different HLA-B27 alleles

B27 allele	HLA-B27 mAb		
	ABC-m3	GS145.2	FD705
B*2701	n.t.	n.t.	+ (29)
B*2702	+ (27,153)[1]	+ (153)	weak (27,153); + (29)
B*2703	-/weak (153)	+ (153)	+ (29.153)
B*2704	n.t.	+ (152)	+ (29)
B*2705	+ (27,150,153)	+ (152,153)	+ (29,150,152,153)
B*2706	+ (153)	+ (153)	+ (29,153)
B*2707	n.t.	n.t.	+ (29)
B*2708	+ (27,150)	n.t.	- (27,150)
B*2709-2727	n.t.	n.t.	n.t.

Table 2: [1] False-positive reactivity of HLA-B27 mAb with other HLA-B antigens
n.t. = not tested

1.2.3 External Quality Assessment

Flow cytometric HLA-B27 screening has to deal with above-mentioned cross-reactivity of HLA-B27 mAb. A biannual EQA scheme for flow cytometric screening of the HLA-B27 antigen is operational in The Netherlands and Belgium since 1995. The results of an EQA scheme should offer an unique opportunity to analyze the cross-reactivity of the commonly used mAb for flow cytometric HLA-B27 screening, i.e., FD705, ABC-m3 and GS145.2, in the setting of a multicenter study. In this way, modifications of protocols for flow cytometric HLA-B27 screening can be proposed to improve the performance of HLA-B27 screening and to meet an extend of standards that are formulated by ASHI and EFI regarding flow cytometric HLA-typing (e.g., viability, labeling of specimens, and reagents).

In this thesis, we (retrospectively) analyzed the results of 7 EQA send-outs to assess the quality of flow cytometric HLA-B27 screening in the Benelux (Chapter 2). Furthermore, we performed further research to compose guidelines toward improvement of flow cytometric HLA-B27 screening, also adhering to the ASHI and EFI guidelines for tissue typing laboratories (Chapter 4 and 5).

1.3 CD34+ CELL ENUMERATION

1.3.1 Clinical background

Since the late 1950's, HPC transplantation has been used to reconstitute hematopoiesis after myeloablative therapies in man (39). Initially, autologous and allogeneic HPC transplants were performed utilizing bone marrow (BM) as a source of HPC (reviewed in 40) but in the last decade, other sources for HPC have been used. Increasingly, peripheral blood stem and progenitor cells (PBSC) are utilized for both autologous and allogeneic transplants (41). In 1998, 95% of autologous transplants and 25% of allogeneic transplants reported to the International Bone Marrow Transplant Registry (Milwaukee, WI) used PBSC (42). A substantial number of patients requiring allogeneic HPC transplants are still unable to find an unrelated donor mismatched for no more than one HLA antigen (43). More recently, umbilical cord blood (UCB) has been shown to be a feasible alternative source of allogeneic HPC (44). Transplantation of HPC from UCB has successfully been used, especially in children (45,46). Also, successful UCB transplants have been reported in adults, in spite of the relatively low HPC dose per kg body weight of the recipient (47). The reconstitution of hematopoiesis takes longer after UCB than after BM or PBSC transplantation, but the incidence and severity of GVHD are lower with UCB transplants, even with HLA-mismatched donors (44,46,48).

The pioneering studies of Berenson *et al* (49) showed that the engraftment potential of BM is contained within cells that bear the CD34 antigen. The CD34 antigen was expressed on a small subset of BM mononuclear cells that in initial studies (50-52) contained the majority of colony-forming-cell (CFC) activity for myelo-erythroid progenitors. After HPC transplantation, hematopoietic recovery is dependent on the number as well as the quality of transplanted CD34+ HPC. In the setting of allogeneic transplantation, several studies agree that faster neutrophil and platelet recovery are related to higher CD34+ cell counts in recipients of BM and PBSC grafts (53-57). In general, 2.0 x 10^6 CD34+ cells/kg patient body weight may be a safe minimum cell dose for both BM and PBSC grafts (58).

When the target CD34+ cell dose has been set, enumeration of CD34+ stem cells has been shown to be useful in the planning of PBSC harvesting (59-62), and the monitoring of various PBSC mobilization strategies (63). Bye and large, peripheral blood counts between 10 and 20 CD34+ cells/µl generally predict aphaeresis yields of 0.5 to 1 x 10^6 CD34+ cells per kg patient body weight per collection day. Peripheral blood counts between 20 and 40 CD34+ cells/µl will predict aphaeresis yields ~1 x 10^6/kg, and counts >40 CD34+ cells/µl typically predict that one single harvest is enough to secure a transplant of 2 x 10^6/kg patient body weight (16,59).

The advent and use of a CD34 monoclonal antibody enabled the detection of these cells by flow cytometry (64). A direct correlation between the CD34 content of peripheral blood in patients mobilized with chemotherapy (with or without growth factors such as granulocyte colony stimulating factor (G-CSF)) and the number of CD34+ cells collected by apheresis was shown. Additionally, the number of transplanted HPC as defined by flow cytometry was shown to correlate better with engraftment post HPC transplantation than that assessed using colony-forming unit (CFU) assays (65). As a result, many centers adopted flow cytometric CD34+ HPC enumeration to monitor the rate of HPC mobilization and to assess the engraftment potential of BM and PBSC harvests.

1.3.2 Technical background

Siena *et al* (64) were the first to describe a flow cytometric method to enumerate CD34+ cells. Initially, this method was based on mononuclear cell enrichment by gradient separation followed by staining with the class I CD34 antibody My10 using indirect immunofluorescence techniques (64). Around 1990, fluorescein isothiocyanate (FITC) conjugated CD34 antibodies became available, enabling the modification of this method into a "whole blood, stain-lyse-wash" method, termed the Milan-Mulhouse protocol (66,67). The gating procedure of this method is based on selection of leukocytes as denominator based on FSC and SSC, excluding debris and aggregates. With the commercial availability of phycoerythrin (PE) conjugates of class II (QBEnd10)

and class III (8G12, 581, and Birma-K3) antibodies, the accuracy and sensitivity of Milan-Mulhouse type protocols were slightly increased due to the higher fluorescence intensity of PE versus FITC (13,67-69), and in case of FITC conjugated class II CD34 mAb, the reduced specific binding affinity (14,70). The first multicolor analytical strategy was developed by Bender *et al* (71) in which CD45 FITC is included as a leukocyte marker in addition to CD34 PE. CD45 staining is used to establish a more stable and precise denominator by including only nucleated white blood cells in the analysis. CD45+ events are then analyzed in a similar way to the Milan-Mulhouse protocol using an isotype control and CD34 staining versus SSC analysis to enumerate CD34+ cells. A sequential Boolean gating approach is the multicolor protocol of the Dutch Foundation for Immunophenotyping in Hemato-Oncology (SIHON) (72). With Boolean gating man can create a combination of any existing gate using various Boolean functions (e.g., AND, OR, NOT). The SIHON approach utilizes the laser dye solution LDS-751, that stains DNA and to a lesser degree RNA, to identify nucleated cells and exclude debris, platelets, and unlysed erythrocytes. The nominator is nucleated cells (i.e., LDS-751-^{bright}). To exclude the effects of Fcγ receptor mediated mAb binding by monocytes and granulocytes from subsequent analyses, PE-conjugated antibodies to CD14 and CD66e are utilized (72).

In order to achieve standardization, the development and use of robust CD34+ cell enumeration methods based on flow cytometry is critical. The results obtained with various enumeration methods are dependent on several variables, such as the sample source, sample processing and data analysis techniques, and require significant laboratory skills. Within UCB, one of the technical challenges is the presence of variable numbers of nucleated red blood cells, which can lead to overestimation of the leukocyte count in dual platform methods. Furthermore, samples taken from the UCB transplants prior to cryopreservation for CD34+ cell enumeration are often more than 24 h old when tested. This situation is associated with increased apoptosis, cell death and formation of cellular debris as compared to fresh samples. Importantly, processing, cryopreservation, and thawing of UCB can have a dramatic effect on the recovery of viable HPC (73). Currently accepted practice in umbilical cord blood transplantation is to estimate the engraftment potential of UCB collections based on total leukocytes (74). However, graft progenitor cell content is a better predictor than total cell count for speed of engraftment after UCB transplantation (75).

Meanwhile, a more robust standardized CD34+ cell enumeration protocol has been developed to measure CD34+ cells accurately in a variety of sources of stem cell products. This method can distinguish viable CD34+ cells from non-viable cells and non-specifically stained debris (14,16,76-78).

1.3.3 Single platform ISHAGE protocol including viability assessment

Absolute CD34+ cell counts are traditionally assessed using a dual platform technique: (i) the flow cytometer provides the CD34+ cell percentages as fractions of a denominator, i.e., WBC or lymphocytes, and (ii) the hematology analyzer provides the absolute WBC count together with a differential count, which must include the denominator. In the late 1990's single platform techniques were introduced: the absolute CD34+ cell counts are directly assessed on the flow cytometer in a precisely determined volume of blood sample. Single platform techniques can either be volumetric (79) or based on counting beads (80,81). By incorporating a known number of fluorescent counting beads in the flow cytometric analysis, an absolute CD34+ cell count can be generated using a single instrument platform. Assessment of the ratio between the number of beads and CD34+ cells counted, together with a lyse-no-wash assay format allows the direct calculation of the absolute CD34+ cell count. The advantage of single platform techniques is the elimination of the need for the second instrument. The International Society of Hematotherapy and Graft Engineering (ISHAGE), now International Society for Cell Therapy (ISCT), had commissioned Sutherland and colleagues to develop a robust flow cytometric assay for CD34+ cell enumeration, now known as the "ISHAGE method" (14). The 2-color single-platform ISHAGE method, endorsed by the British (82), Dutch (78), German (15), and European Working Group on Clinical Cell Analysis (EWGCCA) (77,78,83) guidelines, uses this approach and can be extended to a 3-color assay with viability staining using 7-amino-actinomycin (7-AAD) (76). To eliminate cells other than leukocytes as well as debris from the analysis and to generate a stable denominator, CD34 is counterstained by CD45. This use of CD45 was originally described by Bender *et al* (71). However, primitive blast cells, which generally exhibit similar light scatter properties as lymphocytes, express lower levels of CD45 than lymphocytes (84,85). This dim CD45 expression by the CD34+, SSC^{low} HPC is also included in the gating strategy of the ISHAGE protocol. The sequential Boolean gating strategy of the ISHAGE protocol consists of four sequential steps: first, all the viable leukocytes (i.e., CD45+ ranging from dim to bright, 7-AAD negative) are selected (fig. 1A and 1H); second, CD34+ cells are selected among the leukocytes (fig. 1B); third, $CD45^{dim}$, SSC^{low} HPC are selected among the $CD45^{pos\ to\ dim}$, CD34+ events (fig. 1C); and fourth, any events falling outside the cluster of $FSC^{low\ to\ intermediate}$ HPC are excluded by defining a FSC-SSC lymphocyte-blast gate (fig. 1D). The lower limit of CD45 expression by CD34+ events is verified in comparison to the lower CD45 signals of cellular debris (fig. 1E). Also a region is set on the counting beads, selected by their bright signals in both fluorescence channels (fig. 1E). The lymphocyte-blast gate is verified by selecting lymphocytes defined by their SSC-CD45 characteristics (R5, fig. 1A) to make sure that no lymphocytes are excluded (fig. 1F). Singlet counting beads are included on the basis of their uniform, low FSC signals (fig. 1G) whilst non-viable cells (i.e.,

7-AAD+) already had been excluded from analysis (fig. 1H). As detailed elsewhere, this sequential gating strategy obviates the need for an isotype control (14,76-78).

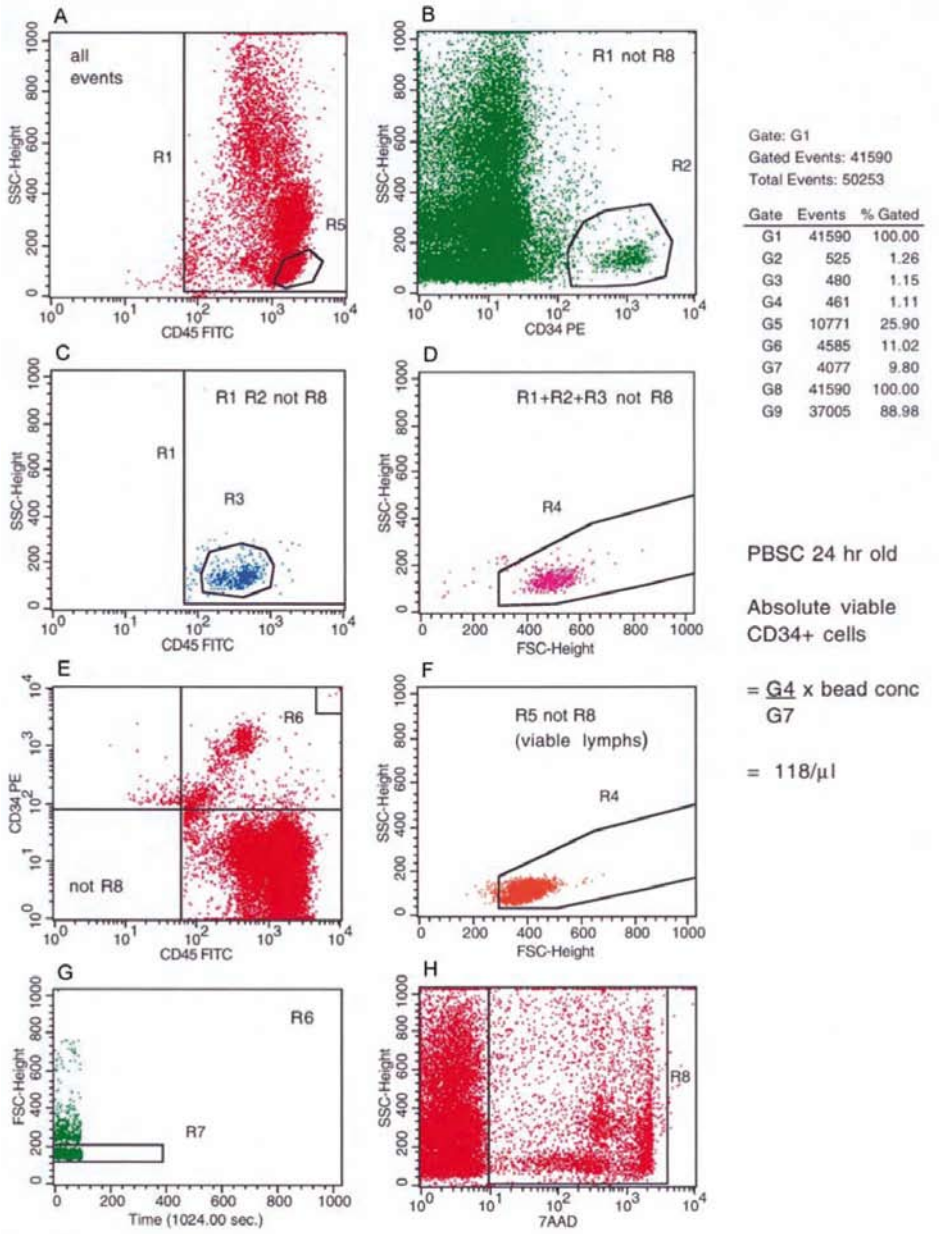

Figure 1: Single platform ISHAGE protocol including viability assessment

The Stem-Kit™ assay, utilizing Flow-Count™ beads (all from Beckman-Coulter, Miami, FL), is a commercial variant of the single-platform ISHAGE protocol as described above. A known number of these beads are added to the test sample at the end of the assay. Brocklebank *et al* (86) described another variation of the single-platform ISHAGE protocol using TruCOUNT™ beads (BDBiosciences), which are provided as a lyophilized pellet in dedicated test tubes. This approach was in their hands highly comparable with the single-platform ISHAGE protocol using Flow-Count™ beads (Beckman-Coulter) (86). The commercial variant of this approach is ProCOUNT™ single-platform kit (BDBiosciences) is based on counting beads (i.e., TruCOUNT™ tubes). The denominator for nucleated cells is a nucleic acid dye (NAD). A threshold is set on nucleated cells. The gating strategy is aimed to identify CD34+ cells; first by gating on NAD^{pos}, $SSC^{low\ to\ intermediate}$ events, and second by gating on $CD45^{neg\ to\ normal\ positive}$ events. Within these events the CD34+ events are analyzed. A similar analysis is run for the isotype-matched control. The TruCOUNT™ counting beads (BDBiosciences) are gated on their FL1, FL2, and FL3 properties.

1.3.4 External quality assessment

To train and implement a standardized protocol for clinical laboratories performing flow cytometric CD34+ cell enumeration, international workshops have been organized. Several national and international EQA schemes for CD34+ cell enumeration have been set up. However, widely varying inter-laboratory coefficients of variation (CVs) have been reported (72,82,87-95). The range of CVs obtained per test sample varied greatly between studies. Interestingly, the stability of the test samples used in multicenter trials appeared to be a complicating factor. In 8 trials, fresh test samples were used. Within various assay methods, inter-laboratory CVs ranged from 8% to 174% (72,87-90,93,95)! In contrast, in 11 trials using stabilized test samples, inter-laboratory CVs ranged from 3% to 89% (82,91,92,94,96). This result may indicate that part of the variation between laboratories in external quality assessment schemes using non-stabilized specimens may be due to variability associated with deterioration of the test samples during transport. Another issue is the assay method itself. The studies using standardized single-platform protocols (i.e., ISHAGE and ProCOUNT™ [BD Biosciences] methods) were among those featuring the lowest inter-laboratory CVs ranged from 3% to 37% (82,94,96), illustrating that single-platform techniques reduced inter-laboratory variation in CD34+ cell enumeration. Within the single platform technologies, the Boolean gating strategy that focuses on the 'true' HPC (i.e., $FSC^{low\ to\ intermediate}$, SSC^{low}, CD45(+), CD34+ cells) and constitutes the core of the ISHAGE protocol (14) and its later modifications (16,76), proved to be indispensable, especially when samples of poor quality have to be analyzed, such as apheresis packs containing significant numbers of dead cells and platelet aggregates, UCB, and cryopreservation

and thawing of transplants (97). Standardization of absolute CD34+ cell counting was pursued by EWGCCA using 3 send-outs of stabilized blood samples containing 150 - 200 CD34+ cells/µl (92). After each send-out, the 24 participating laboratories were debriefed and advised where appropriate. The variation between laboratories, as measured by the CV of the median results of triplicate assays, decreased from 23% to 11%, whilst the intra-laboratory variation, as measured by the CV of triplicate assays, was <5% for 39% of laboratories during the first send-out, which increased to 65% during the third send-out (94). The SIHON also endorsed the ISHAGE protocol, and introduced it to 36 participating laboratories through a similar scheme of 3 trials.

To assess the effect of multiple variables on CD34+ cell enumeration, we analyzed the results of 9 EQA send-outs in the Benelux countries (Chapter 6). Therefore, robust multivariate regression, divergent to the statistics performed in most other studies, was used. We addressed two aspects of the quality of the CD34+ cell enumeration tests: bias (i.e. systematic differences) and variability (i.e. random differences). Furthermore, the EQA send-outs were analyzed for their educational aspects (Chapter 6).

1.4 LYMPHOCYTE SUBSET ENUMERATION

1.4.1 Clinical background

Enumeration of the major T-lymphocyte subsets (i.e., CD4+ and CD8+ T cells) yields important information for diagnosis and monitoring of immunocompromised patients. The discovery of human immunodeficiency virus (HIV) in the 1980's as the causative agent of the destruction of CD4+ T lymphocytes leading to the acquired immunodeficiency syndrome (AIDS) was the major drive behind the evolution of flow cytometry from research tool to routine diagnostic technique (reviewed in 98). As known, the pathogenesis of AIDS is largely attributable to the decrease in the number of CD4+ T cells (99-102). Progressive depletion of CD4+ T cells is associated with an increased likelihood of severe HIV disease and an unfavorable prognosis (103-106). CD4+ T-lymphopenia is also associated with opportunistic infections in individuals infected with the human immunodeficiency virus (HIV) (107) following hematopoietic stem cell transplants (108) and is also a risk factor for skin cancers in renal transplant recipients (109).

Obtaining accurate and reliable measures of CD4+ T cells is essential in assessing the immune system and managing the health care of persons infected with HIV (110-113). For example, measurement of CD4+ T-cell levels has been used to establish decision points for initiating prophylaxis for opportunistic infections and for initiating

and monitoring antiretroviral therapy (114-119). CD4+ T-cell levels are also a criterion for categorizing (i.e., category 1: greater than or equal to 500 cells/μL ; category 2: 200-499 cells/μL ; and category 3: less than 200 cells/μL) HIV-related clinical conditions according to classification system of the Centers for Disease Control (CDC) for HIV infection and surveillance case definition of AIDS among adolescents and adults (120). These categories guide clinical and therapeutic actions in the management of HIV-infected adolescents and adults. The increased need for standardized approaches and quality control procedures in the clinical setting prompted the development of guidelines for CD4+ T-cell enumeration, which also cover other topics (e.g., specimen collection, specimen transport, maintenance of specimen integrity, specimen processing, laboratory safety, quality control, quality assurance, and data analysis) (6).

Next to the CD4+ T cells, the CD8+ T cells are also of interest. CD8+ T cells suppress HIV-1 replication. The CD8+ T cells can be activated and increase inversely in numbers relative to CD4+ T cells with disease progression (121). Furthermore, CD8+ T-lymphocytosis is a hallmark of the immune response to primary cytomegalovirus (CMV) and Epstein-Barr virus infection in healthy carriers (122,123) and is associated with recovery from reactivated CMV infection in renal and SCT recipients (124,125). Furthermore, lymphocyte immunophenotyping is an indispensable part for the diagnostic and therapeutic decision-making in acute lymphoblastic and myeloid leukemias (ALL and AML), lymphomas, and chronic lymphoproliferative disorders (126).

1.4.2 Technical background

In the early 1990's, dual-color assays were used to stain density gradient-isolated mononuclear cell suspensions or white blood cells (WBC) in a "stain, lyse & wash" technique. During list mode data analyses, lymphocytes were distinguished from other WBC subsets on the basis of their bright expression of CD45 and negativity for CD14, followed by "backgating" on their FSC and SSC characteristics (127). This approach performs well on samples with relatively normal proportions of lymphocytes, but light scatter gates on lymphopenic samples frequently suffer from heavy contamination with nonlymphoid cells (128). Later on, the advent of additional fluorochromes made 3- and 4-color assays accessible for the clinical laboratory. This advance prompted the development of improved strategies to select lymphocytes. The "T-gating" method was based on counter-staining CD4 and CD8 with CD3 as marker to select the T cells in combination with SSC (129). Alternatively, with the 'dual-anchor' approaches, lymphocytes are selected first on the basis of bright CD45 expression and low SSC, followed by the selection of T cells on the basis of CD3 positivity, counterstained with CD4 and/or CD8 (130-132). These gating strategies were subsequently adopted in CDC (133), National Institute for Allergic and Infectious Diseases (NIAID), Division of AIDS (DIADS) (134) and British Committee for Standards in Haematology (BCSH)

(5) guidelines. Absolute T-cell subset counts are traditionally assessed using a dual platform technique, but in the late 1990's single platform techniques were introduced. Side by side comparisons of single-platform techniques with 'double-anchor' gating strategy, with dual-platform assays combined with "backgating" strategy revealed that the first approach was able to reduce inter- and intra-laboratory variation in absolute T-cell subset counting (135,136). The use of single-platform technology was already recommended by the BCSH guidelines (5) and was incorporated in the 2003 update (6) of the CDC guidelines.

Unlike CD4+ T-cell enumeration, lymphocyte immunophenotyping relies less on identification and enumeration of a single, physiological cell population. In contrast, this diagnostic procedure requires the identification of one or more abnormal populations based on the profile composed of the intensity of expression of multiple markers. The specific patterns generated by flow cytometry in these conditions provide important diagnostic information, which is of potentially prognostic value for some cases of leukemia or lymphoma. As a result of the complexity of this field, initial guidelines for this testing were less uniform than those for CD4+ T-cell or CD34+ cell enumeration (8-11). There are several attempts to address critical issues in this area, such as the definition of marker panels suitable for detecting different subtypes of acute leukemia (12,137) including the biphenotypic leukemias (11,138), and the selection of marker combinations useful for minimal residual disease detection (139).

1.4.3 External Quality Assessment

Various national organizations, for example (i) the NIAIDS-DIAIDS and the College of American Pathologists (CAP) in the United States, (ii) the National HIV Immunology Laboratory (Health Canada), Canadian Quality Management Programme - Lab Services (QMP-LS), and the Canadian HIV Trials Network (CTN) in Canada, (iii) UK NEQAS for Leucocyte Immunophenotyping in the United Kingdom, (iv) the Dutch Foundation for Quality Control of Medical Laboratory Diagnosis (SKML), and (v) the Belgian Association for Analytical Cytometry (BVCA/ABCA) operate an EQA with similar frequency to assess inter-laboratory and intra-laboratory immunophenotyping performance and to assist laboratories with the implementation of 'state of the art' technologies. Data obtained in these EQA's have been analyzed and published (140-142). For example, the Fall 2003 survey of approximately 500 laboratories by the CAP, using 3 samples of stabilized whole blood with CD4 percentages ranging from 17 to 52, showed that the CV between laboratories ranged on average from 4% to 8% for % CD3+, CD4+ T cells expressed as proportion of lymphocytes. In the same survey, 220 laboratories performing absolute CD4+ T-cell counts achieved inter-laboratory CVs from 7% to 16%. Here, CD3 was not included in the data analysis for lineage gating of T cells, and contamination of the gated lymphocytes with CD3-, CD4dim monocytes

may have contributed to the relatively large inter-laboratory variation. In contrast, the laboratories participating in the surveys of the QMP-LS and the CTN have widely adopted dual-anchor gating and single platform techniques. As a result, this - much smaller - group of laboratories consistently achieves inter-laboratory CVs from 5% to 10% for absolute CD4+ T-cell counts. The European experience has been similar with two reports showing inter-laboratory variations in the same order of magnitude as observed in the American studies (140,143). Laboratories using less sophisticated gating strategies (e.g., CD45, CD14-based backgating or simple light scatter-guided gating on lymphocytes) were up to 7.4 times more likely to fail an EQA exercise (140). In addition to the EQA schemes available to North American, United Kingdom, and European centers, the QASI program (Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS) was established in 1997 to meet performance assessment for immunophenotyping laboratories in countries where such service is not available (141). A total of 47 countries participate in this survey, including many from African, South American and the Asia Pacific region. Since the start of this program, the inter-laboratory CV decreased from 7.2% to 4.7% and from 14.2% to 8.8% for percent and absolute CD4+ T-cell counts, respectively. Interestingly, over two thirds of participants used relatively simple two-color methods based on the selection of lymphocytes as CD45bright,SSClow (142). The general consensus is now that this "panleucogating" approach significantly increases the reliability of absolute CD4 counts (141,142). However, no consensus exists yet on the issue whether or not single platform should be preferred above dual platform methods, particularly in view of the fact that single platform assays may not be practical or affordable in their current format for use in resource-poor countries (142).

To assess the effect of multiple variables on CD3+ T-cell, CD4+ T-cell, CD8+ T-cell, NK-cell, and CD19+ B-cell enumeration, we analyzed the results of 10 EQA send-outs in the Benelux countries (Chapter 7). Therefore, robust multivariate regression, divergent to the statistics performed in most other studies, was used. We addressed two aspects of the quality of the T- and B-cell subset enumeration: bias (i.e. systematic differences) and variability (i.e. random differences). Furthermore, the EQA send-outs were analyzed for their educational aspects (Chapter 7).

1.5 SCOPE OF THIS THESIS

In this thesis, we analyze the impact of standardization efforts on clinical cell analysis by flow cytometry with special attention to the EQA programs, which currently are in operation in the Benelux countries. Flow cytometric HLA-B27 screening, CD34+ cell enumeration, CD4+ T-cell enumeration, and lymphocyte immunophenotyping were the assays of interest. The continued advances being made in fluorochrome technology, hardware and automated software for flow cytometry, should allow for improvements but also provides challenges due to increasing complexity of the assays. We focus on the improvement of the assays as well as the educational impact of the EQA on the performance of the participants.

The first part of this thesis covers the educational impact of the EQA and trends toward improvement in flow cytometric HLA-B27 screening. We chart the performance of the participating sites in identifying HLA-B27^{pos} and HLA-B27^{neg} samples. Therefore, data of the first seven send-outs (i.e., 28 samples) to which 36 to 47 laboratories participated was analyzed. Additionally, the results of this EQA offered the unique opportunity to analyze the cross-reactivity of the commonly used anti-HLA-B27 mAb for flow cytometric HLA-B27 typing in the setting of a multicenter study (Chapter 2). The cross-reactivities observed in this study were mostly but not fully in agreement with those observed in other studies. Therefore, we performed a comprehensive analysis of the cross-reactivity patterns of five anti–HLA-B27 mAb that were commercially available for flow cytometric HLA-B27 screening in 2001 (Chapter 3). Based on the results of these 2 studies we propose a Standard Operation Procedure (SOP) to improve the flow cytometric HLA-B27 screening assay (Chapter 4). The ASHI and EFI standards for flow cytometric HLA typing, with which accredited tissue typing laboratories must comply, are also taken into account. Therefore, we studied the feasibility of a long-term stable whole blood preparation for use as positive and negative HLA-B27 control cells for both daily and longitudinal quality control, for reagent validation, and for EQA purposes (Chapter 5).

The second part of this thesis covers the educational aspects and trends toward improvement in flow cytometric CD34+ cell enumeration. We analyzed the results of nearly 3,000 assays, in the context of methodological information provided in questionnaires that were issued with each send-out. We were interested in identifying factors affecting the outcome of CD34+ cell enumeration, and in identifying factors influencing the variability of this assay. Furthermore, we focused on the efficacy of the EQA program to reduce inter-laboratory variation of CD34+ cell enumeration as a function of time (Chapter 6).

The third part of this thesis covers the educational aspects and trends toward improvement in flow cytometric CD4+ T-cell enumeration and lymphocyte immunophe-

notyping. We analyze the results of nearly 23,000 assays, in the context of methodological information provided in questionnaires that were issued with each send-out. As for CD34+ cell enumeration, we were interested in identifying factors affecting the outcome of lymphocyte immunophenotyping and in identifying factors influencing the variability of this assay. In addition, we focused on the efficacy of the EQA program to reduce inter-laboratory variation of lymphocyte immunophenotyping as a function of time (Chapter 7).

Finally the results are discussed and summarized (Chapter 8). The role of the above-mentioned EQA schemes, which has been primarily educational, for further improvements as well as laboratory accreditation purposes is discussed. With these regulatory aspects in view, suggestions for further improvement of the EQA schemes themselves are made (Chapter 8).

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Wilfried H.B.M. Levering
Rene van den Beemd
Jeroen G. te Marvelde
Wil A.M. van Beers
Herbert Hooijkaas
Kees Sintnicolaas
Jan W. Gratama

EXTERNAL QUALITY ASSESSMENT OF FLOW CYTOMETRIC HLA-B27 TYPING

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ABSTRACT

Introduction

A biannual external quality assurance (EQA) scheme for flow cytometric typing of the HLA-B27 antigen is operational in The Netherlands and Belgium since 1995. We report here on the results of the first seven send-outs to which 36 to 47 laboratories participated.

Methods

With the send-out, four specimens from blood bank donors, who had been typed for HLA Class I antigens by complement-dependent cytotoxicity, were distributed. Subtyping of the HLA-B27 allele was performed by PCR-SSP. Ten samples were HLA-B27^{pos} (all HLA-B*2705) and 18 were HLA-B27^{neg}. For flow cytometry, the most widely monoclonal antibody (MoAb) used was FD705, followed by GS145.2 and ABC-m3. The majority of laboratories used more than 1 anti-HLA-B27 MoAb for typing.

Results

The HLA-B27^{pos} samples were correctly classified as positive by the large majority of participants (median 95%; range 85% to 100% per send out); some participants considered further typing necessary and misclassification as negative was only sporadically seen. The classification of HLA-B27^{neg} samples as negative was less straightforward. Ten samples were correctly classified as such by 97% (82% to 100%) of the participants, whereas 64% (range 53% to 70%) of the participants classified the remaining eight samples as HLA-B27^{neg}. There was no significant prevalence of a particular HLA-B allele among these eight "poor concordancy" samples as compared to the ten "good concordancy" samples. Inspection of the reactivity patterns of the individual MoAb with HLA-B27^{neg} samples revealed that ABC-m3 showed very little cross-reactivity apart from its well-known cross-reactivity with HLA-B7, whereas the cross-reactivity patterns of GS145.2 and FD705 were more extensive. The small sample size (n=18) and the distribution of HLA-B alleles other than HLA-B27 did not allow assignment of specificities to these cross-reactions.

Conclusions

Finally, we showed that standardized interpretation of the combined results of two anti-HLA-B27 MoAb reduced the frequency of false-positive conclusions on HLA-B27^{neg} samples. In this series, the lowest frequency of false-positive assignments was observed with the combination of the FD705 andABC-m3 MoAb.

INTRODUCTION

HLA-B27 is a serologically defined antigen of the HLA-B locus. The demonstration of the strong association between HLA-B27 and ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system, dates already back to 1973 (1,2). This association between HLA and disease, which is one of the few that are close to 100%, has been confirmed in many studies and extended to all major ethnic groups (reviewed in 3). In fact, HLA-B27 is common to the entire group of seronegative spondyloarthropathies, which also include Reiter's disease, subgroups of intestinal and psoriatic arthropathies, subgroups of juvenile rheumatoid arthritis, and acute anterior uveitis (4). Consequently, screening for HLA-B27 is used for the differential diagnosis of patients with lower back pain and is, therefore, performed in many clinical diagnostic laboratories. In addition to the joints, the heart has been identified as an important target for HLA-B27-associated disease. Specifically, atrioventricular conduction blocks and isolated aortic regurgitation are strongly associated with HLA-B27 (5). A variety of methods has been developed to type for HLA-B27. The most common methods are the complement-dependent cytotoxicity (CDC) assay (6), flow cytometry (FCM) (7), molecular typing based on DNA amplification such as the polymerase chain reaction (PCR) with sequence specific primers (PCR-SSP) (8), and PCR with sequence specific oligonucleotides (PCR-SSO) (9,10). Other methods such as typing using cytotoxic T-lymphocytes as cellular reagents (11), isoelectric focusing (12), and the serological assessment of HLA-B27 positivity using soluble HLA-antigens in serum (13) have also been described but are not used in routine diagnostic practice.

Flow cytometric HLA-B27 typing is complicated by the fact that HLA-B27 is member of a large cross-reacting group (CREG). CREGs consist of HLA antigens that share common epitopes (14). HLA-B27 is part of the HLA-B7 CREG that also includes HLA-B7, B13, B22, B40, B41, B42, B47, and B48. Cross-reactivity of HLA typing reagents, in particular human sera, is commonly encountered. Several murine monoclonal antibodies (MoAb) have been raised against HLA-B27, such as FD705 (One Lambda, Canoga Park, CA), GS145.2 (Becton Dickinson Biosciences [BDB], San Jose, CA), and ABC-m3 (available through Behring [Brussels, Belgium], Serotec [Oxford, UK], Silenus Laboratories [Hawthorn, Australia] and Immunotech [Marseille, France]) (15–17). Unfortunately, HLA-B27-specific MoAb cross-react too, usually with antigens within the HLA-B7 CREG (summarized in Table 1).

In an attempt to control for such cross-reactivities, the American Society for Histocompatibility and Immunogenetics (ASHI) and the European Federation for Immunogenetics (EFI) have included the following recommendation in their standards for serological HLA Class I typing in general (18,19): Each HLA-A, B or C antigen should be defined

by at least two sera, if both are operationally monospecific. If sera are operationally multispecific, at least three partially nonoverlapping sera should be used.

An additional problem is that the reactivity of anti-HLA-B27 MoAb with the different HLA-B27 alleles (as defined by DNA typing) may vary. Currently, 15 HLA-B27 alleles are known and have been assigned by the WHO Nomenclature Committee for Factors of the HLA System (20). An overview of published reactivities with HLA-B27 alleles is shown in Table 2.

Table 1: Crossreactivities of anti HLA-B27 MoAb: summary of published studies

Crossreactivity with HLA-B antigens	anti HLA-B27 MoAb		
	FD705	ABC-m3	GS145.2
B7	23	15,22,23,24,25,26,27,28	15,16,23,29
B13		26	
B22		25,26,27	16
B37	23	23,27	16,23
B39			16
B41			16
B42		27	
B44	23	23,27	23
B55(22)			23
B57	23		
B73		27	
none	7,15,17,22	26	30

Table 1: Numbers refer to references in Literature Cited

Table 2: Reactivity of various HLA-B27 monoclonal antibodies with different HLA-B27 alleles

B27 allele	HLA-B27 MoAb		
	FD705	ABC-m3	GS145.2
B*2701	+ (7)	nt	nt
B*2702	weak (27,34); + (7)	+ (27,34)	+ (34)
B*2703	+ (7,34)	-/weak (34)	+ (34)
B*2704	+ (7)	nt	+ (30)
B*2705	+ (7,28,30,34)	+ (27,28,34)	+ (30,34)
B*2706	+ (7,34)	+ (34)	+ (34)
B*2707	+ (7)	nt	nt
B*2708	- (27,28)	+ (27,28)	nt

Table 2: B*2709-2715: no data available. nt = not tested

A biannual external quality assessment (EQA) scheme for HLA-B27 typing has been set up for Dutch and Belgian laboratories in 1995 by the Dutch Flow Cytometry Working Party under the coordinated auspices of the Foundation for Quality Control in Medical Immunology (SKMI), the Foundation for Quality Control of Hospital Laboratories (SKZL), both in The Netherlands, and the Belgian Association for Cytometry (BVC/ABC). We report here on the results of the first seven send-outs with special attention to false-positive results on HLA-B27^{neg} samples due to cross-reactivities of the used anti-HLA-B27 MoAb.

MATERIALS AND METHODS

External Quality Assessment Scheme

This biannual EQA scheme was offered by the Foundation for Quality Control in Medical Immunology (SKMI), the Foundation for Quality Control of Hospital Laboratories (SKZL), and the Belgian Association for Cytometry (BVC/ABC) to all Dutch and Belgian laboratories that routinely performed HLA-B27 typing for diagnostic purposes. For each EQA send out, the participants were provided with and were requested to process peripheral blood samples from four blood bank donors (denoted 1 to 28 in this and the following sections). The coordinating laboratories (Laboratory for Histocompatibility and Immunogenetics, Blood Bank Rotterdam and Department of Immunology, University Hospital and Erasmus University Rotterdam) collected and aliquotted the samples, and had them dispatched by overnight express mail at ambient temperature (i.e., 15° to 25°C) to arrive at the participating laboratories the following day at 9 a.m., i.e., within 24 h after venipuncture. The participants had to keep the samples at room temperature and process them on the day of receipt. The participants were requested to submit printed output of list-mode data analyses and to report the following items for each sample and each HLA-B27 MoAb: (a) a summary of the methodology used and printed output of list mode data analyses; (b) whether the reaction was positive or negative; and (c) the final interpretation, i.e., whether the samples were HLA-B27 positive, negative, or needed additional tests. After reporting of the results to the coordinating laboratories and completion of the analyses by those laboratories a confidential report was sent (to each participant) with comments on its own results in comparison to those of the anonymous other participants. In addition, the results were presented and discussed at a yearly plenary meeting.

Reference Methods for HLA-B27 Typing

Gold standards.

All samples were typed for HLA-A, B and Cw using the standard CDC test (6), while HLA-B27 DNA high resolution typing was performed using PCR-SSP (Dynal, Oslo, Norway) (8). These assays were performed at the Laboratory for Histocompatibility and Immunogenetics.

Flow cytometry.

As reference, flow cytometric HLA-B27 was performed at the Department of Immunology using an extended panel of MoAb consisting of three anti-HLA B27 MoAb (clones FD705, ABC-m3 [Serotec] and GS145.2), an anti-HLA-B7 MoAb (clone BB7.1; Serotec), an anti-HLA-Bw4 MoAb (clone 0624; Biotest, Dreieich, Germany) and an anti-HLA-Bw6 MoAb (clone 1001; Biotest). All MoAb, except anti-HLA-Bw4 and Bw6, were conjugated with fluorescein isothiocyanate (FITC). The GS145.2 MoAb was supplied as a mixture with CD3 MoAb (Anti-Leu 4 conjugated with phycoerythrin; BDB) plus reference beads to define the FITC fluorescence (FL) intensity threshold for classification as positive. As isotype control MoAb, mouse IgG1 (clone X40) and mouse IgG2a (clone X39; both from BDB) were used.

One hundred microliters of undiluted blood was added to 20 ml MoAb (mixture), incubated for 20 min at room temperature (RT). The erythrocytes were then lysed using FACS Lysing Solution (BDB) during 10 min at RT, after which the remaining leukocytes were washed once with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA). Following centrifugation for 5 min at 400 3g, the cell pellet was resuspended in 0.5 ml PBS containing 1% paraformaldehyde.

Prior to data acquisition the flow cytometer (FACScan; BDB) was set up and calibrated according to a standard procedure (21). In addition, a separate instrument set-up according to the manufacturer's procedure (HLA-B27 screening kit; BDB) was made to accommodate analysis of the samples stained with the GS145.2 1 Anti-Leu 4 MoAb mixture. The samples were acquired within 2 h after completion of the staining procedure. Data of 10,000 events were collected in list mode. List mode data of the samples stained with the GS145.2 1 Anti-Leu 4 MoAb mixture were analyzed with dedicated software (BDB). For data analysis of the other samples, the lymphocytes were selected on the basis of their forward and sideward light scatter characteristics. The FL1 marker to discriminate between positive and negative FITC FL signals was then set on the basis of the isotype control staining.

Data Processing, Reporting, and Statistical Analyses

The participating laboratories were requested to submit their data on questionnaires which were checked for inconsistencies and entered into a database by the SKMI data center (Diagnostic Center SSDZ, Delft, The Netherlands). The questionnaire also included an inventory of local methods, addressing sample preparation, MoAb used, and methods for flow cytometric data analysis, to analyze the effects of these technical features on the HLA-B27 typing results. Following each send-out, each participant received a confidential report with (comments on) its results in comparison to those of the anonymous other participants. Statistical analyses and graphical representations of the data were prepared using Stata software (Stata Corp., College Station, TX). The significance of 2 3 2 tables was calculated using Fisher's exact test (two-sided).

RESULTS

Distributed Specimens and Response Rate

Forty-seven laboratories participated in the autumn 1995 survey. For the 1996, 1997, and 1998 spring and autumn surveys these numbers were 36, 39 and 43, respectively. The response rate ranged between 91% and 100% in all seven surveys. Each survey consisted of four specimens. A total of 10 HLA-B27^{pos} and 18 HLA-B27^{neg} samples were distributed. Detailed typing results by the "gold standards," i.e., complement-dependent cytotoxicity and PCR-SSP assays, are shown in Table 3.

Local Protocols for Flow Cytometric HLA-B27 Typing

A few laboratories used PCR-SSP instead of flow cytometry: 0 in 1995, 1 in 1996, 4 in 1997, and 3 in 1998. The results obtained using PCR-SSP were not included in this analysis. Table 4 gives an overview of the local protocols for sample preparation, and number and clones of MoAb used. The proportion of laboratories using >1 anti-HLA-B27 MoAb increased from 50% in 1995 to 86% in 1997; the recommendations of the EQA scheme organizing committee to do so may have contributed to this increment. However, this proportion fell to 68% in 1998, which was partly due to the entry of some laboratories using only one MoAb and partly due to the fact that other laboratories reduced their MoAb panel to one antibody. Over the years, the most widely used MoAb clone was FD705, followed by GS145.2 and ABC-m3, respectively.

EQA Scheme Results: Overall Conclusions

An overview of the overall conclusions of the participants per sample (i.e., "negative," "further typing required," or "positive") is shown in Figure 1. The 10 HLA-B27^{pos} samples were correctly classified as positive by the majority of participants (median

Table 3: Results of reference laboratory HLA typing of HLA-B antigens of samples 1–28 used in EQA*

Sample	HLA-B antigens (CDC)[a]				HLA-B allele
No.	HLA-B	HLA-B	HLA-Bw4	HLA-Bw6	(DNA)
1	8	**27**	+	+	B*2705
2	*7*	blank	-	+	-
3	*7*	**27**	+	+	B*2705
4	*44*	blank	+	-	B*4402
5	*44*	*57*	+	-	B*4402
6	38	*39*	+	+	-
7	*44*	38	+	-	B*4403
8	38	*39*	+	+	-
9	*7*	*55*	-	+	-
10	**27**	blank	+	-	B*2705
11	*44*	*73*	+	+	B*4403
12	*44*	47	+	-	B*4403
13	**27**	blank	+	-	B*2702,2705
14	*57*	blank	+	-	-
15	**27**	51	+	-	B*2705
16	38	51	+	-	-
17	51	**27**	+	-	B*2705
18	*37*	blank	+	-	-
19	51	blank	+	-	-
20	**27**	*37*	+	-	B*2705
21	*7*	*37*	+	+	-
22	*44*	*37*	+	-	B*4402
23	58	**27**	+	-	B*2705
24	*57*	**27**	+	-	B*2705
25	18	48	-	+	-
26	*41*	blank	-	+	-
27	60	blank	-	+	-
28	**27**	35	+	+	B*2705

Table 3: * HLA typing was performed using the CDC test and DNA typing as previously described. [a] + = antigen/allele present; - = antigen/allele absent; blank = second HLA-B antigen not detected; no proof for HLA-B homozygosity
x = cross-reactive HLA antigen as reported in the literature (summarized in Table 1).

95%, range 85% to 100%). A minority of participants considered further typing necessary (median 3%, range 0% to 13%), and samples were only sporadically incorrectly classified as negative (median 0%, range 0% to 5%). Ten HLA-B27^{neg} samples were correctly classified as negative by .75% of the participants, i.e., samples 4, 6, 7, 9, 11, 16, 19, 25, 26 and 27 (median 97%, range 82% to 100%). False positive conclusions

Table 4: Overview of local protocols for flow cytometric HLA-B27 typing

		Year			
Feature		1995	1996	1997	1998
Number of registered laboratories		47	38	43	45
Number of responding laboratories		47	36	39	43
HLA-B27 typing by PCR		0	1	4	3
HLA-B27 typing by FCM		47	35	35	40
Sample preparation	Lysed whole blood	38	31	33	35
	Not stated	9	4	2	5
HLA-B27 MoAb	FD705	35	28	31	32
	ABC-m3	18	16	17	20
	GS145.2	15	17	20	22
Additional MoAb	HLA-B7	5	8	9	11
	HLA-Bw4	5	4	4	3
	HLA-Bw6	4	4	2	3
	HLA-B7/40	1	1	1	1
Number of HLA-B27 MoAb	1	23	8	5	13
	>1	23	25	30	27
	Not stated	1	2	0	0

were only incidentally drawn on four of these samples and were not reported at all on the other six samples. However, the conclusions on the remaining eight HLA-B27^{neg} samples (i.e., samples 2, 5, 8, 12, 14, 18, 21, and 22) were more diverse. These samples were correctly classified as negative by ,75% of the participants (median 64%, range 53% to 70%), while 30% (26% to 35%) considered further typing necessary and 6% (0% to 13%) classified the samples

incorrectly as positive. There was no significant prevalence of a particular HLA-B antigen among these eight "poor concordancy" HLA-B27^{neg} samples as compared to the other ten "good concordancy" HLA-B27^{neg} samples. Of interest, all eight "poor concordancy" HLA-B27^{neg} samples were positive for one or two HLA-B antigens other than B27 to which cross-reactivity of any of the three MoAb (FD705, ABC-m3, and GS145.2) was reported. In contrast, only five of the ten "good concordancy" HLA-B27^{neg} samples expressed one or two of these "non-HLA-B27" antigens (P = 0.04).

EQA Scheme Results: Performance of Individual Anti-HLA-B27 MoAb

In this section we focus on the performance of the FD705, ABC-m3, and GS145.2 MoAb on HLA-B27^{neg} samples as the interpretation of their reactivities with HLA-B27^{pos} samples was straightforward (Fig. 1). The results of these three MoAb on the 18 HLA-

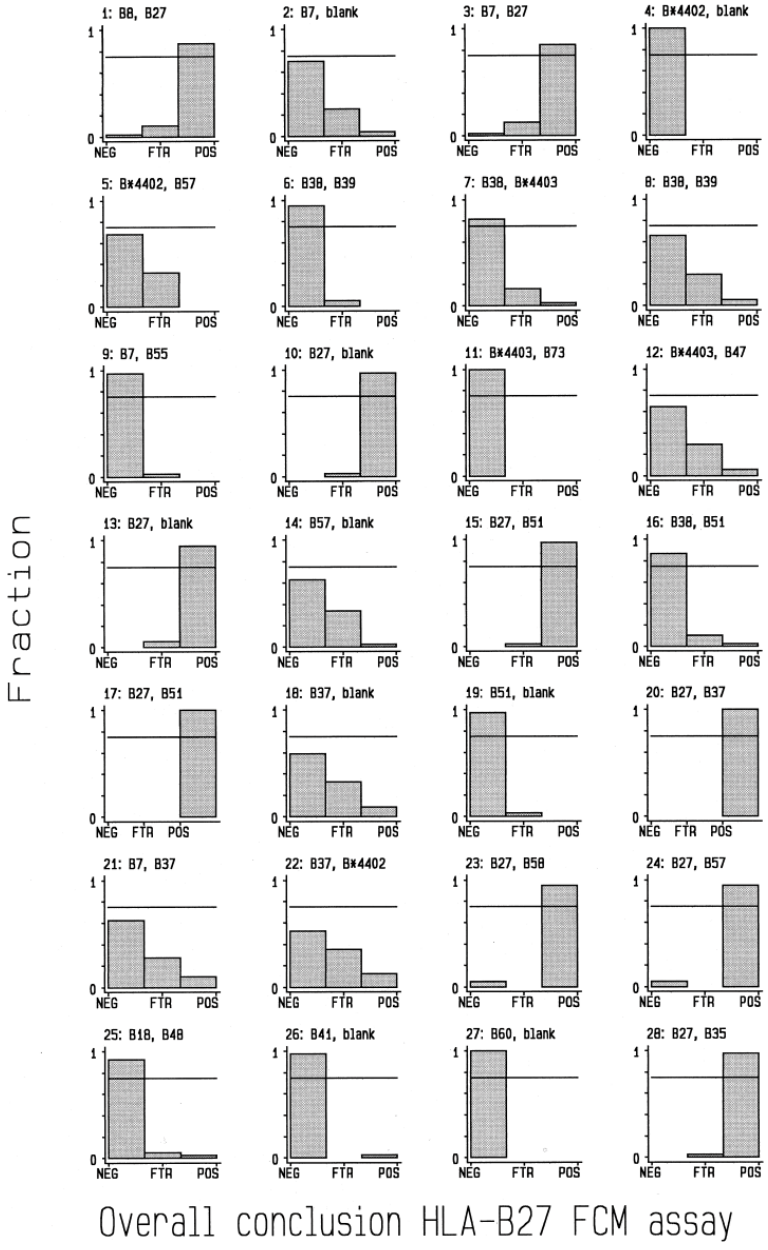

Figure1: Overall conclusions of all participating laboratories on 28 samples distributed in seven EQA scheme rounds. The laboratories were requested to report their conclusions as "negative," "further typing required," or "positive." The fraction of laboratories reporting a given conclusion is plotted on the y-axis; a reference line is drawn at the 0.75 level in each panel (see further Results). The HLA-B typing result obtained by complement-dependent cytotoxicity is plotted above each panel.

B27^{neg} samples were plotted per sample in Figure 2. We classified a sample as showing significant crossreactivity if <75% of the laboratories reported a negative result (indicated by the horizontal line in each panel of Fig. 2). In addition, flow cytometric histograms showing the reactivities of these MoAb with the eight "poor concordance" samples are shown in Figure 3.

The FD705 MoAb showed significant cross-reactivity on 9 of the 18 HLA-B27^{neg} samples, i.e., samples 5, 7, 8, 12, 14, 16, 18, 21, and 22 (Fig. 2).

Cross-reactivity typically presented as a relatively dim fluorescent signal, i.e., mean FL intensity falling in the second of 4 log decades versus the third log decade for HLA-B27^{pos} samples and the first log decade for the isotype control MoAb staining (Fig. 3). The proportion of false positive results was even ≈75% on samples 12 and 22. The

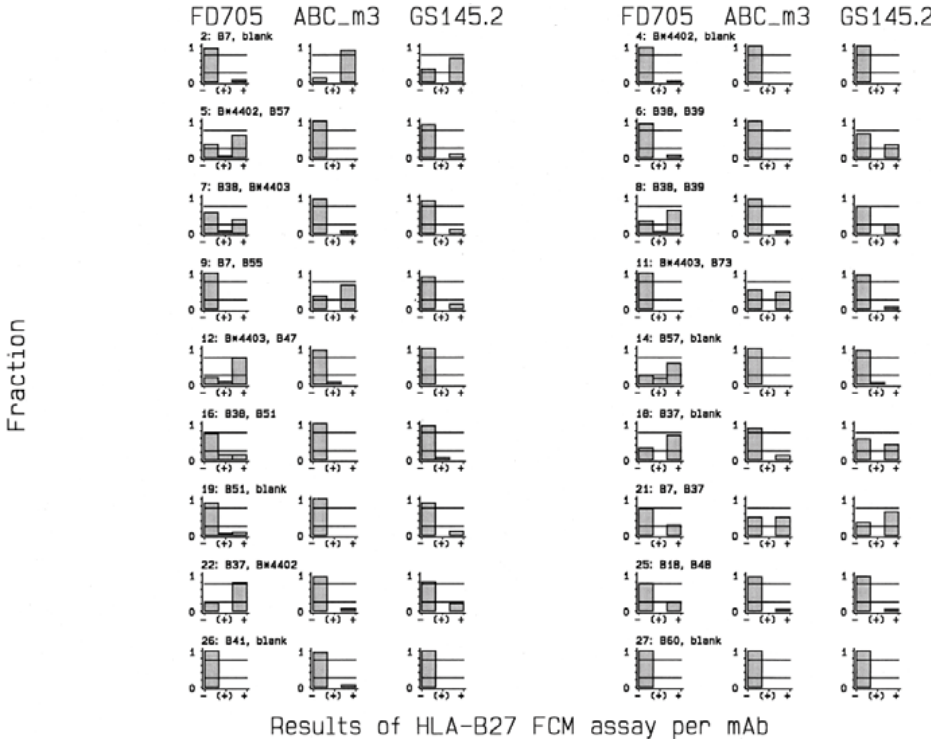

Figure 2: Results obtained by all participating laboratories on 18 HLA-B27^{neg} samples stratified by anti-HLA-B27 MoAb used (i.e., FD705, ABC-m3, and GS145.2). Results are expressed as "2", negative; "(1)", weakly positive; "1", unambiguously positive (for examples see Fig. 3). The fraction of laboratories reporting a given result is plotted on the y-axis; reference lines are drawn at the 0.25 and 0.75 levels in each panel (see further Results). The HLA-B typing result obtained by complement-dependent cytotoxicity is plotted above each panel.

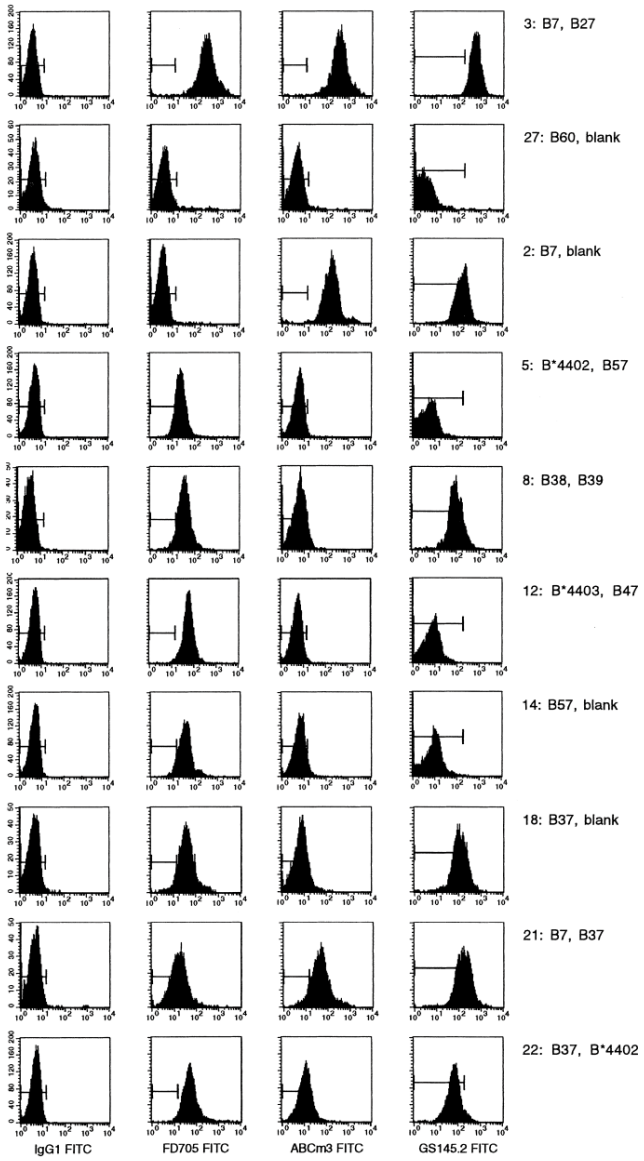

Figure 3: FITC fluorescence histograms obtained with the anti HLA-B27 MoAb FD705, ABC-m3, and GS145.2 (middle and right columns) and IgG1 isotype control MoAb (left column) on the HLA-B27^{pos} sample 3 (upper row), the non-crossreactive, HLA-B27^{neg} sample 27 (second row) and the 8 samples with the least concordant results between the laboratories (samples 2, 5, 8, 12, 14, 18, 21, and 22; see Results). Left three panels: Data shown were obtained after gating on lymphocytes (see Materials and Methods); the FL marker was set at the foot of the isotype control MoAb histogram. Right panel: Data shown were obtained after gating on CD3^{pos} lymphocytes, and the FL marker was placed in a predefined intensity level after calibration of the FL scale with the BDB HLA-B27 screening kit and software.

cross-reactivity observed on samples 18, 21, and 22 may have been due to HLA-B37 as there were no HLA-B27^{neg},37^{pos} samples with <75% negative classifications. By the same token, HLA-B57 may explain the cross-reactivity on samples 5 and 14. Sample 12 was the only HLA-B47^{pos} sample, which precludes any firm conclusions as to HLA-B47 as a source of crossreactivity. No straightforward explanation of cross-reactivity was possible for the remaining samples 7, 8, and 16. The antigens HLA-B38, B39, B44 (3x B*4402 and 3x B*4403) and B51 were both expressed by samples with <75% negative classifications as by those with >75% negative classifications. Apparently, these HLA-B antigens are an unlikely target for cross-reactivity with FD705 MoAb in our study.

The ABC-m3 MoAb showed in this series much less cross-reactivity than the FD705 MoAb: 4 of the 18 samples, i.e., samples 2, 9, 11 and 21, had <75% negative results reported (Fig. 2). Again, cross-reactivity presented as a relative dim signal (i.e., mean FL intensity in the second log decade) while sample 2 (HLA-B7, blank) even showed a stronger signal (i.e., mean FL intensity in the third log decade, only slightly less than the HLA-B27^{pos} samples; Fig. 3). Three of the four cross-reactive samples (i.e., 2, 9, and 21) were HLA-B7^{pos} versus 0 of the 14 non-cross-reactive samples (P = 0.005). The fourth sample, 11, was HLA-B44^{pos},73^{pos} (B*4403). Sample 11 was the only HLA-B73^{pos} sample, which precludes any firm conclusions as to HLA-B73 as a source of cross-reactivity. HLA-B*4403 was an unlikely source of cross-reactivity, as two other HLA-B*4403^{pos} samples (i.e., 7 and 12) were classified as HLA-B27^{neg} by >75% of the laboratories using ABC-m3.

The GS145.2 MoAb showed significant cross-reactivity with 5 of the 18 HLA-B27^{neg} samples, i.e., samples 2, 6, 8, 18, and 21 (Fig. 2). Samples 2 and 18 were homozygous for HLA-B7 and B37, respectively, indicating cross-reactivity of GS145.2 with these antigens. This is in concordance with the cross-reactivity observed with sample 21 (HLAB7, 37). The negative reactions with two heterozygous samples (sample 9: B7, 55; sample 22: B44, 37) may be explained by the dosage effect (i.e., either one HLA-B7 or HLA-B37 allele present) and does not exclude cross-reactivity of GS145.2 with HLA-B7 and B37. Samples 6 and 8 expressed both HLA-B38 and B39. HLA-B38 is an unlikely explanation for cross-reactivity as two other HLA-B38^{pos} sample (i.e., samples 7 and 16) had <25% false-positive scores with GS145.2. HLA-B39 may have been a target for cross-reactivity, but no further HLA-B39^{pos} samples were present in our series to support this contention.

Finally, we investigated retrospectively whether or not the standardized interpretation of the combined results of two anti-HLA-B27 MoAb would have contributed to a better

typing result than the interpretation of results obtained using only a single MoAb. This analysis (Fig. 4) was based on the individual results per MoAb and sample reported by each participant (Fig. 2). The best classification was obtained with the following protocol for data interpretation. If both MoAb yielded an unambiguously positive result (i.e., "1" in Fig. 2), the conclusion was "positive." For all other combinations of unambiguously positive, weakly positive (i.e., "(1)" in Fig. 2) and negative (i.e., "2" in Fig. 2), the conclusion on the combined result was "negative." In this way, a conclusion "further typing required" was avoided. On all HLA-B27^{pos} specimens, any combination of two of the three anti-HLA-B27 MoAb, i.e., FD705, ABC-m3, and GS145.2, led to the conclusion "positive" on the basis of the data from all laboratories. The situation for the 18 HLA-B27^{neg} samples was, again, less straightforward (Fig. 4). The combination

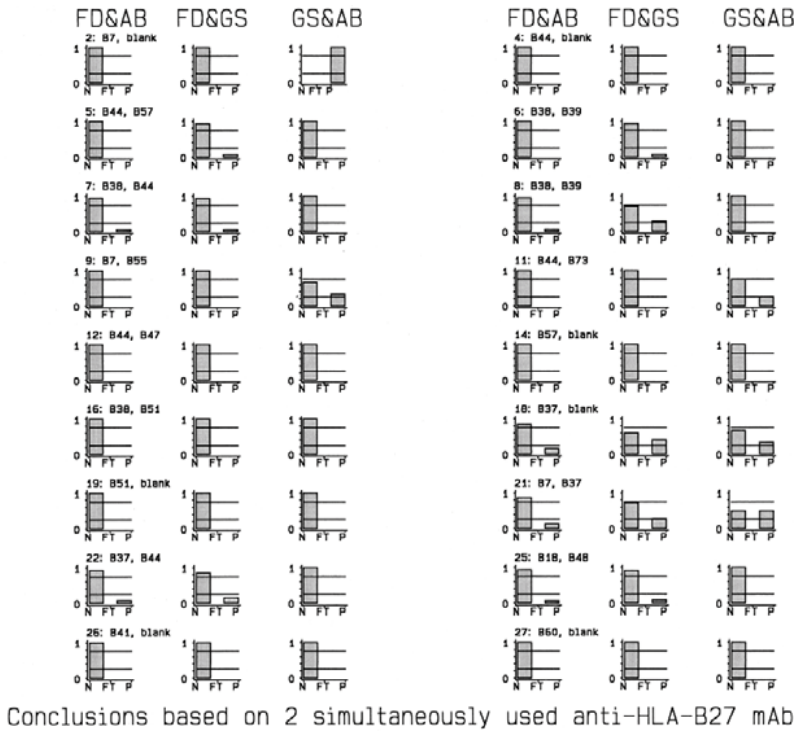

Figure 4: Conclusions, drawn retrospectively, on the basis of the combined results of two anti-HLA-B27 MoAb reported by all participating laboratories on 18 HLA-B27^{neg} samples. The conclusions are abbreviated as "N," HLA-B27 negative; "P," HLA-B27 positive; the conclusion "FT" (further typing required) was not incorporated in the standardized interpretation (for details see Results). The fraction of laboratories on whose results a given conclusion was drawn is plotted on the y-axis; reference lines are drawn at the 0.25 and 0.75 levels in each panel (see Results). The HLA-B typing result obtained by complement-dependent cytotoxicity is plotted above each panel.

FD705 and ABC-m3 performed best: the conclusion "negative" was obtained on the basis of the results of >75% of the participants on all 18 HLA-B27^{neg} samples. Occasional false-positive conclusions were drawn on six samples. The other two combinations, FD705 and GS145.2 and GS145.2 and ABC-m3, yielded less satisfactory results. Using FD705 and GS145.2, 15 of the 18 HLA-B27^{neg} samples were classified as negative on the basis of the results of >75% of the participants, and false-positive conclusions were drawn on eight samples. The combination GS145.2 and ABC-m3 yielded the conclusion "negative" on the basis of the results of >75% of the participants on only 13 of the 18 samples. Even worse, a false-positive assignment was made to sample 2 (B7, blank) on the basis of the results of all participants.

The false-positive classifications on the remaining four samples were quite frequent, i.e., based on the results of ≥25% of the participants. These results show that standardized interpretation of the combined results of two anti-HLA-B27 MoAb can indeed reduce the frequency of false-positive conclusions on HLA-B27^{neg} samples. In this series and with this interpretation protocol, the lowest frequency of false-positive assignments was observed with the combination of the FD705 and ABC-m3 MoAb.

DISCUSSION

We report here on our 3.5-year experience with external quality assessment of flow cytometric HLA-B27 in a scheme serving 36 to 47 clinical diagnostic laboratories in The Netherlands and Belgium. The performance of the laboratories in identifying HLA-B27^{pos} samples as such was good: a median of 95% (range 85% to 100%) classified such samples correctly. Evidently, this result is restricted to HLA-B*2705 as only samples with this subtype were available for distribution. The results obtained with the 18 HLA-B27^{neg} samples were less concordant. Cross-reactivity of the used anti-HLA-B27 MoAb with other HLA-B antigens plays a major role in this respect. Ten HLA-B27^{neg} samples were correctly classified as such by a median of 97% (range 82% to 100%) of the participants. In contrast, the remaining HLA-B27^{neg} samples were correctly classified by only 64% (53% to 70%) of the participants. The published results of studies addressing such cross-reactivity of the FD705, ABC-m3, and GS145.2 MoAb are summarized in Table 1. Interestingly, all samples of the second group of 8 HLA-B27^{neg} donors were positive for one or more of these cross-reacting antigens versus only five of the first group of ten HLA-B27^{neg} donors. This result indicates that cross-reactivity of the typing reagents with HLA-B antigens other than B27 is an important cause of false-positive classifications.

The results of this EQA scheme offered the unique opportunity to analyze the cross-reactivity of the commonly used MoAb for flow cytometric HLA-B27 typing, i.e., FD705,

ABC-m3 and GS145.2, in the setting of a multicenter study. Initially, the FD705 MoAb was considered monospecific by several groups (7,15,22). Two of these reports (7,15) conclude that FD705 does not crossreact with the HLA-B27 CREG, but specify only incompletely which antigens of the HLA-B27 CREG had been tested. The third report (22) evaluated FD705 against a relatively low number (i.e., ten) of HLA-B27^{neg} donors that did not include HLA-B37 or B57. In contrast, our results are consistent with those of Hoffmann *et al*. (23), i.e., that FD705 cross-reacts with HLA-B37 and B57. However, our data are not in agreement with Hoffmann's observations that FD705 cross-reacts with HLA-B7 and B44.

Expectedly, our results confirmed the widely observed cross-reactivity of the ABC-m3 MoAb with HLA-B7 (15,22–28). Our suggestion that ABC-m3 might cross-react with HLA-B73 is consistent with Coates' results (27). In contrast, our data do not confirm the reported cross-reactivities of ABC-m3 with HLA-B37 and HLA-B44 (23,27).

The GS145.2 MoAb has been reported to cross-react with HLA-B7 by several groups (15,16,23,29). Our data, although not conclusively, appear to support these results. Interestingly, our notion that GS145.2 may crossreact with HLA-B37 and B39 has also been reported (16,23). Our data do not support Hoffmann's conclusion that GS145.2 cross-reacts with HLA-B44. On the other hand, the total absence of cross-reactivity of GS145.2 with other HLA-B antigens, as reported by Chou (30), was not supported by our data either.

The Standards for Histocompatibility Testing of the American Society for Histocompatibility and Immunogenetics (ASHI) (18) and the European Federation for Immunogenetics (EFI) (19) recommend, for HLA-typing by flow cytometry, that "a single monoclonal antibody may be used to define an antigen provided its monospecificity has been sufficiently verified by local testing." As none of the three commonly used MoAb (i.e., FD705, ABC-m3, and GS145.2) are monospecific for HLA-B27, we explored whether or not the combined use of two of these MoAb would have improved the results of flow cytometric HLA-B27 typing. This retrospective analysis revealed that the combination of FD705 and ABC-m3 MoAb performed best on the 28 distributed samples: correct positive assignments by all laboratories to all 10 HLA-B27^{pos} samples, and correct negative assignments by >75% of laboratories to all 18 HLA-B27^{neg} samples. The latter result may be explained by the fact that FD705 and ABC-m3 have less overlapping cross-reactivities than FD705 and GS145.2 (i.e., HLA-B37) and ABC-m3 and GS145.2 (i.e., HLA-B7). A major advantage of the combination of FD705 and ABC-m3 is that the presence of HLA-B7 will not lead to false positive test results. ABC-m3 will cross-react with HLA-B7 (Table 1), while cross-reactivity of FD705 with HLA-B7 has only been reported once (23) and was not apparent in our study. However, it must be mentioned that the cells used for the EQA scheme were highly selected on the basis of their expected cross-reactivities and therefore do not represent the general

population. In this context it is interesting to recall that the shared cross-reactivity of FD705 and GS145.2 with HLA-B37 may have explained the false-positive results with a HLA-B37^{pos},57^{pos} sample in an EQA scheme of the ASHI and the College of American Pathologists (31).

Based on this analysis, we propose the following strategy for decision making in flow cytometric HLA-B27 typing: (i) use two HLA-B27 MoAb, i.e., FD705 and ABC-m3; (ii) a sample is classified HLA-B27^{pos} when both MoAb react positively; (iii) in all other cases a sample is classified HLA-B27^{neg}. This policy has the advantage that no further typing is necessary, but carries the risk of occasional false-positive and false-negative reactions. False-positive reactions may occur when two cross-reactive antigens for both MoAb are present, e.g., HLA-B7 and HLA-B37. False-negative reactions may occur with certain rare HLA-B subtypes such as HLA-B*2708 (27,28).

In conclusion, our 3.5-year experience with HLA-B27 EQA scheme has shown that flow cytometry can identify HLA-B27^{pos} samples (i.e., the subtype HLA-B*2705, prevalent in the caucasoid population), but that cross-reactivity of the commonly used HLA-B27 MoAb is a major problem that frequently leads to false-positive test results. HLA-B27 typing by flow cytometry may be further improved by (i) application of quantitative flow cytometry to standardize the distinction between relatively dim (cross-reactive) and strong (true positive) signals (32,33); (ii) complete documentation of the reactivity of all currently defined HLA-B27 alleles (i.e., B*2701 to B*2715) with the available anti-HLA-B27 MoAb; and (iii) rigorous analysis of the crossreactivity patterns of these MoAb with HLA-antigens other than HLA-B27. We feel that there will remain a place for cost-effective and simple screening methods for selected HLA alleles, such as HLA-B27, in the clinical diagnostic laboratory. Flow cytometry is one such method that, when appropriately performed and interpreted, can produce high-volume, cost effective and rapid turn around of selected clinical testing. However, DNA-based typing methods, which nowadays constitute a gold but costly standard, are already commercially available for HLA-B27 typing. As molecular biology will become more commonly applied by clinical diagnostic laboratories in the near future, we foresee that flow cytometric HLA-B27 screening will lose some ground to molecular typing.

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Wilfried H.B.M. Levering
Henk Wind
Kees Sintnicolaas
Herbert Hooijkaas
Jan W. Gratama

FLOW CYTOMETRIC HLA-B27 SCREENING: CROSS-REACTIVITY PATTERNS OF COMMERCIALLY AVAILABLE ANTI–HLA-B27 MONOCLONAL ANTIBODIES WITH OTHER HLA-B ANTIGENS

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ABSTRACT

Background

Some 50 clinical laboratories in the Benelux perform flow cytometric HLA-B27 screening and participate in the Benelux external quality assessment scheme operational since 1995. Results from this scheme indicate that cross-reactivity of HLA-B27 monoclonal antibodies (mAbs) is a major problem.

Methods

We analyzed cross-reactivity patterns of commercially available mAbs for HLA-B27 screening. Three clones of HLA-B27 mAb (ABC-m3, n = 3; FD705; and GS145.2) from five manufacturers were evaluated. Test cells were selected as to express HLA-B antigens with known serologic cross-reactions (HLA-B7, B12, B13, B16, B17, B22, B37, B40, B41, B42, B47, and B48). Cells without B27 cross-reactive antigens (B5, B8, B14, B15, B21, and B35) and cells positive for B27 were included as controls. All tests were performed and interpreted as recommended by the manufacturers. Cross-reactivity was defined as increased fluorescence intensity in comparison with the baseline reactivity observed with the corresponding immunoglobulin G isotype control mAb.

Results and Conclusions

All mAbs tested showed cross-reactivity, ranging from weak (±) to strong (+), with different antigens and different degrees of intensity. ABC-m3: (±) B12, B16, B17, B41, B47, and B48 and (+) B7, B13, B22, B37, B40, and B42; GS145.2: (±) B13, B17, B22, B40, and B47 and (+) B7, B16, B37, B42, and B48; FD705: (±) B12, B13, B16, and B48 and (+) B17, B37, and B47. If one mAb had been used for HLA-B27 screening, ABC-m3 would have yielded nine false-positive B27 assignments, FD705 would have yielded seven, and GS145.2 would have yielded two. This problem largely can be avoided by the combined use of two different mAb clones. The combination of FD705 and GS145.2 yielded the best results, with one false-positive HLA-B27 assignment among the 99 HLA-B27$^-$ samples of this highly selected panel.

INTRODUCTION

Since 1973, a strong association between HLA-B27 and ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system, has been demonstrated (1–3). It has become evident that HLA-B27 is common to the entire group of seronegative spondyloarthropathies, which include Reiter's disease, subgroups of intestinal and psoriatic arthropathies, subgroups of juvenile rheumatoid arthritis, and acute anterior uveitis (4). In addition, the heart has been identified as a target for HLA-B27–associated disease; certain atrioventricular conduction blocks and isolated aortic regurgitation have been strongly associated with HLA-B27 (5). Therefore, screening for HLA-B27 is performed in many clinical diagnostic laboratories.

In addition, HLA-typing is performed with the complement-dependent cytotoxicity (CDC) assay (6), whichis commonly used in tissue typing laboratories. Various manufacturers offer CDC-based typing trays containing sera selected for HLA-B27 typing. However, for general diagnostic laboratories, the CDC assay is too time consuming and expensive if used only for HLA-B27 screening, because it requires specialized knowledge and extensive quality-control procedures. Therefore, alternative methods were developed for HLA-B27 screening: (a) flow cytometry (7), (b) DNA-based typing using polymerase chain reaction (PCR) assays based on sequence-specific primers (8) or sequence-specific oligonucleotides (9–11), and (c) enzyme-linked immuno-sorbent assays (12). Among these alternative methods, flow cytometric HLA-B27 screening is used most widely, although DNA-based typing is gaining ground. For example, of the participants in the external quality assessment scheme for HLA-B27 screening in the Netherlands and Belgium, none performed DNA-based typing in 1995 versus 11% in 2000 (13). Flow cytometric HLA-B27 screening is hampered by the fact that HLA-B27 belongs to the large HLA-B7 cross-reacting group (CREG). Within the HLA-B7 CREG, HLA-B13, B22, B27, B40, B41, B42, B47, and B48 share common epitopes with HLA-B7 (14,15). In addition, cross-reactivities of HLA-B27 typing reagents with non–HLA-B7 CREG antigens, i.e., HLA-B12, B16, B17, B37, and B73, have been reported (13,16–18). The cross-reactivities of human sera used in CDC assays are well known. However, murine monoclonal antibodies (mAbs) against HLA-B27 also cross-react (Table 1). In 2001, these mAbs included ABC-m3 (available through Serotec, Oxford, UK; Immunotech, Marseille, France; and Silenus Laboratories, Hawthorn, Australia) (16,19,20), GS145.2 (Becton Dickinson [BD] Biosciences, San Jose, CA), and FD705 (One Lambda, Canoga Park, CA). The combined analysis of the results of the studies summarized in Table 1 identified HLA-B7 as the major cross-reacting antigen for all three mAb clones, followed by HLA-B12 and HLA-B37. However, the combined data of these studies showed inconsistent patterns with respect to the cross-reactivities of HLA-B27 mAbs with other HLA-B antigens (Table 1). To optimally advise the partici-

pants of our external quality assessment scheme, we performed a systematic study of cross-reactivities of the five mAbs against HLA-B27 that were commercially available in 2001 by using a panel of cell donors selected to express the range of known HLA-B antigens with documented serologic cross-reactivities with HLA-B27.

Table 1: Cross-reactivities of anti–HLA-B27 monoclonal antibodies: summary of published studies

Cross-reactivity with HLA-B antigens	Anti-HLA-B27 monoclonal antibody[a]		
	ABC-m3	GS145.2	FD705
B7	13,17-19,24-28	13,16,17,19,29	17
B12	13,17,18	13,17	13,17
B13	27		
B17			13,17
B22	18,26,27	16,17	
B37	13,17,18	13,16,17	13,17
B39(16)		13,16	
B41		16	
B42	18		
B73	13,18		
None		30	8,19,20,24

Table 1: [a] Numbers refer to referenced publications.

MATERIALS AND METHODS

Blood Donors

From the HLA-typed donor registry of the Sanquin Blood Bank South West Region, 115 donors were requested, after informed consent, to donate 18 ml blood each. All samples were typed for HLA-A, -B, and -Cw with the standard CDC test (7). In addition, HLA-B locus low resolution typing by PCR with sequence specific primers (Olerup-SSP, Saltsjo¨baden, Sweden) was performed. To analyze cross-reactivities of the anti–HLA-B27 mAbs, we selected 67 donors that were heterozygous for any of the known HLA-B antigens with documented serologic crossreactivities (i.e., HLA-B7, B12, B13, B16, B17, B22, B27, B37, B40, B41, B42, B47, and B48) and expressed an HLA-B antigen with no known serologic cross-reactivity with HLA-B27. These donors are referred to as HLA-Bx,n in this and the following sections. Specifically, eight B7,n, eight B12,n (i.e., eight B44,n), four B13,n, six B16,n (i.e., three B38,n and three B39,n), three B17,n (i.e., three B57,n), seven B22,n (i.e., seven B55,n), seven B37,n, seven B40,n (i.e., five B60,n and two B61,n), seven B41,n, three B42,n, six B47,n, and one B48,n donors were tested. Further, 25 donors presumably homozygous for any of the known HLA-B

antigens with documented serologic crossreactivities were investigated. These donors are designated HLA-Bx,-. Specifically, we tested four B7,-, three B12,- (i.e., three B44,-), four B13,-, three B16,- (i.e., two B38 and B39 and one B39,-), three B17,- (i.e., three B57,-), one B22,- (i.e., one B56,-), one B37,-, four B40,- (i.e., three B60,- and one B61,-), and two B41,- donors. As positive controls, 12 HLA-B27 heterozygous (B27,n) and four HLA-B27 homozygous (B27,-) donors were used; as negative controls, seven donors expressing non–cross-reactive HLA-B antigens were included.

Reagents

We studied the HLA-B cross-reactivity patterns of the following anti–HLA-B27 mAbs:

1. HLA-ABC-m3 fluorescein isothiocyanate (FITC; clone ABC-m3; catalog no. MCA116F, Serotec) and mouse immunoglobulin (Ig) G2a negative control FITC (catalog no. MCA929F, Serotec).
2. IOTest HLA-B27 FITC (clone ABC-m3, Immunotech) plus HLA-B7 PE (catalog no. PN IM1502, Beckman Coulter, Miami, FL) and isotypic control IgG2a FITC-IgG1 phycoerythrin (PE) dual-color reagent (catalog no. PN IM1255, Beckman Coulter).
3. Com-B27 FITC (clone ABC-m3, catalog no. 987058010) and control IgG2a FITC (code: 09CONT02E; both from Silenus Laboratories).
4. HLA-B27 Screening Kit (catalog no. 340183, BD Biosciences) containing anti–HLA-B27 mAb clone GS145.2 FITC mixed with the CD3 PE mAb clone SK7.
5. HLA-B27 FITC (clone FD705, catalog no. B27F50X) and mouse IgG2b FITC-conjugated isotype control (catalog no. G2BF50; both from One Lambda).

Each donor was tested once against the full set of reagents. Tests were performed within 24 h after venipuncture.

Flow Cytometry

Assays were performed on 57 occasions over a 300-day period. The FACScalibur instrument was set up by using Calibrite beads and FACScomp software (all from BD Biosciences). In addition, the appropriate positioning of the FL1 window of analysis in the sample space was verified with reference beads (RCP-60, Spherotech, Libertyville, IL). On all 57 test days, the FL1 channel was calibrated by using Quantum FITC low-level beads (catalog no. 824; Bangs Laboratories, Fishers, IN). The so-called FL1 primary performance parameters (21), derived from FL1 calibration regression analyses, were entered into Levey-Jennings plots. Thus, the FL1 primary performance parameters of the instrument were, by and large, within the proposed limits of acceptability (21). The average residual was less than 3%, the FL1 detection threshold was fewer than 1,000 molecules of equivalent soluble fluorochrome (MESF), the FL1

coefficient of response was near the lower limit of the specifications for a 4-log decade amplifier (i.e., between 59 and 69), and the FL1 zero-channel value remained near the upper limit of the acceptable range of 200 MESF. In this way, the FL1 channel was validated for quantitative measurements of fluorescence intensity (expressed as FITC MESF). In addition, a separate instrument setup according to the manufacturer's procedure (HLA-B27 Screening Kit, BD Biosciences) was used to analyze the samples stained with the GS145.2 + SK7 mAb mixture (BD Biosciences).

Each reagent was tested according to the manufacturer's instructions. For HLA-B27 mAbs except clone GS145.2 (HLA-B27 Screening Kit, BD Biosciences), 50 µl of undiluted blood was added to the recommended amount of mAb and incubated for 20 min at room temperature (RT) in the dark. Then 2 ml of ammoniumchloride solution was added, followed by vortexing and incubation for 10 min at RT in the dark to lyse the erythrocytes. After centrifugation for 5 min at 400g, the remaining leukocytes were washed twice in 2 ml of phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin. After removing the supernatant, the cell pellet was resuspended in 0.25 ml of PBS containing 1% paraformaldehyde. Using the HLA-B27 Screening Kit, 50 µl of undiluted blood was added to 30 µl of GS145.2 FITC/CD3 PE mixture, followed by vortexing and incubation for 15 min at RT in the dark. Then 2 ml of 1x FACS

Lysing Solution was added, followed by vortexing and incubation for 10 min at RT in the dark to lyse the erythrocytes. After centrifugation for 5 min at 400g, the remaining leukocytes were washed in 2 ml of PBS. After removing the supernatant, the cell pellet was resuspended in 0.25 ml of PBS containing 1% paraformaldehyde. For acquisition, the flow cytometer was set up by using Cali-

BRITE beads and FACSComp software (BD Biosciences). A minimum of 15,000 events was acquired. Flow cytometry was performed within 2 h after completing the various staining procedures. For data acquisition, a "live gate" was set on the forward and side light scatter characteristics of lymphocytes. Within this live gate, 3,000 lymphocytes were counted. Data of ungated events were collected in list mode. For data analysis, the lymphocytes were selected by their forward and side light scatter characteristics. The FL1 marker to discriminate between positive and negative signals was set by using the FITC signal of the recommended isotype control mAb for each HLA-B27 typing reagent. When interpreting the results, a true positive test result was defined by a nonoverlapping FITC signal relative to the negative control. Two exceptions were made to comply with the manufacturers' instructions. First, the FL1 marker for the Immunotech ABC-m3 mAb was set immediately to the left of the curve of the FITC signal of an HLA-B27 heterozygous positive sample. Second, list mode data of the samples stained with the GS145.2 + SK7 mAb mixture (HLA-B27 Screening Kit) were collected and analyzed with dedicated software (BD Biosciences). Briefly, a forward scatter FL2 gate was defined to include at least 50% of the CD3+ lymphocytes. Placement of the FL1

threshold between negative and positive was guided by the calibration beads of the kit. Subsequently, the median fluorescence intensity of the GS145.2 FITC signal was calculated for the gated CD3+ lymphocytes. Samples with a median FL1 signal result equal or higher than the threshold were considered positive for HLA-B27.

Data Management and Statistical Analyses

For each of the 115 donors, the following data were collected for each mAb after gating on the lymphocytes: (a) the median channel number of the isotype control histogram, (b) the median channel number of the anti–HLA-B27 mAb histogram, and (c) the qualitative interpretation of the result (i.e., negative or positive). The data collected under steps (a) and (b) were also expressed in MESF units by using linear regression analysis based on the calibration results of the FL1 channel of each experimental session. Reactivity of HLA-B27 typing reagents with lymphocytes from HLA-B27+ donors was classified as absent if the FL signal did not exceed 2,000 MESF, as minor if the FL signal ranged between 2,001 and 10,000 MESF, as moderate if the FL signal ranged between 10,001 and 50,000 MESF, and strong if the FL signal exceeded 50,000 MESF. For the HLA-B27 Screening Kit, the conclusion obtained with the dedicated software was used. Conclusions were formulated on the basis of the combined results of any combination of two HLA-B27 screening reagents tested.

We developed cross-reactivity scores to summarize the cross-reactivities of the five anti–HLA-B27 mAbs, as detailed below (Table 2). These analyses were performed separately for HLA-B heterozygous and homozygous donors. These scores were based on the gene frequency of each HLA-B allele in the Caucasian population and multiplied by a factor derived from the median FL intensity observed for each mAb against that HLA-B antigen. This factor was 0 for FL intensities no greater than 2,000 MESF, 0.5 for FL intensities between 2,001 and 10,000 MESF, 1.0 for FL intensities between 10,001 and 50,000 MESF, and 2.0 for FL intensities exceeding 50,000 MESF. For example, the median FL signal obtained with the Serotec ABC-m3 mAb on HLA-B7 heterozygous donors was 56,631 MESF and the HLA-B7 gene frequency was 12.39%. This result produced a cross-reactivity score of 2.0 x 12.39 = 24.78. For each mAb, the cross-reactivity scores for HLA-B7 to B48 were then computed by adding the scores for the individual HLA-B antigens. Thus, the weakest cross-reactivity is reflected by the lowest cross-reactivity score.

We also developed conclusion scores to summarize the failure of the Silenus ABC-m3 mAb, the HLA-B27 Screening Kit, and the FD705 mAb to generate the appropriate "negative" conclusion on HLA-B27–negative donors expressing HLA-B7 CREG antigens. These analyses were performed separately for HLA-B heterozygous and homozygous donors. When the results of single mAbs were interpreted, conclusions were *negative*

Table 2: Summary of cross-reactivities of anti–HLA-B27 monoclonal antibodies with other HLA-B alleles[a]

HLA-B allele	n Tested		ABC-m3 (SER)		ABC-m3 (IOT)		ABC-m3 (SIL)		GS145.2 (BDB)		FD705 (ONL)		Allele gene frequency[b]
	Bx,n	Bx,-	Bx,n	Bx,-	Bx,n	Bx,-	Bx,n	Bx,-	Bx,n	Bx,-	Bx,n	Bx,-	
NXR	7	NA	(+)	NA	(+)	NA	(+)	NA	-	NA	-	NA	NA
B7	8	4	++	++	+	+	(+)	+	+	+	-	-	12.39
B12	8	3	(+)	(+)	(+)	(+)	(+)	(+)	-	-	(+)	(+)	11.10
B13	4	4	(+)	+	(+)	+	(+)	(+)	(+)	(+)	-	(+)	3.52
B16	6	3	(+)	+	(+)	(+)	(+)	(+)	+	+	(+)	(+)	4.54
B17	3	3	+	(+)	(+)	(+)	(+)	(+)	-	(+)	(+)	+	4.40
B22	7	1	+	++	+	+	+	+	(+)	(+)	-	-	2.67
B37	7	1	+	+	(+)	(+)	(+)	+	+	+	(+)	+	1.32
B40	7	4	(+)	+	(+)	(+)	(+)	(+)	(+)	(+)	-	-	6.95
B41	7	2	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-	1.11
B42	3	0	++	ND	+	ND	+	ND	+	ND	-	ND	0.02
B47	6	0	(+)	ND	(+)	ND	(+)	ND	(+)	ND	+	ND	0.24
B48	1	0	(+)	ND	(+)	ND	(+)	ND	+	ND	(+)	ND	0.01
Cross-reactivity score[c]			47	55	32	33	26	32	25	27	11	15	

Table 2: [a] Median fluorescein isothiocyanate FL1 intensities were scored as follows: -, ≤2,000 MESF; (+), 2,001–10,000 MESF; +, 10,001–50,000 MESF; ++, >50,000 MESF. In case one donor was investigated, these test results were interpreted instead of the median. BDB, BD Biosciences; Bx,-, HLA-B homozygous donor; Bx,n, HLA-B heterozygous donor; IOT, Immunotech; ONL, One Lambda MESF, molecules of equivalent soluble fluorochrome; NA, not applicable; ND, not done; NXR, non–cross-reactive with HLA-B antigens (i.e., any HLA-B allele other than HLA-B27 and those listed in the table); SER, Serotec; SIL, Silenus.

[b] Allele frequency assessed within the German Caucasian population (n = 14,835) (30).

[c] For computation of cross-reactivity scores, see Materials and Methods.

or *further typing required* (FTR) instead of *positive* according to the guidelines of the American Society for Histocompatibility and Immunogenetics (ASHI) (22) and European Federation for Immunogenetics (EFI) (23). In the present study, the conclusion score for each HLA-B CREG antigen was computed by the fraction of donors designated FTR and multiplied by the gene frequency of that HLA-B allele. For example, we reached the conclusion FTR for 38% of eight HLA-B7 heterozygous donors by using the Silenus ABC-m3 mAb, which produced a conclusion score of 0.38 x 12.39 = 4.70. For each mAb, the conclusion scores for HLA-B7 to B48 were then computed by adding the scores for the individual HLA-B antigens. Thus, the highest accuracy of the conclusions obtained with a given mAb is reflected by the lowest conclusion score. Similarly, conclusion scores were computed for any combination of two mAbs by using the Silenus

ABC-m3 mAb, the GS145.2 mAb, and the FD705 mAb. Conclusions were negative if both mAbs yielded a negative result, positive if both mAbs yielded a positive result, and FTR if both mAbs yielded conflicting results. The conclusion scores were then computed as described above, with the addition that, in case of a false positive conclusion, the score was doubled as compared with the conclusion FTR. Standard statistical tests were performed as detailed in Results.

RESULTS

Control mAb Test Results

The results obtained with the Serotec, Immunotech, Silenus, and One Lambda FITC-conjugated isotype control mAbs are shown in Figure 1. The median values of the histograms obtained with the Serotec, Immunotech, and Silenus control mAbs fell within the first log decade (i.e., < channel 255), which corresponded with fewer than 2,000 MESF units. The One Lambda control mAb

showed not only show higher background staining but also more variation between donors than did the other three control mAbs. The median values of the One Lambda histograms ranged between 1,800 and 7,000 MESF, i.e., some extended into the left half of the second log decade. Of note, the higher background reactivity of the One Lambda control mAb appeared to correlate with the donors' HLA-B types (Fig. 1, lower left panel). The differences between One Lambda control mAb reactivities as a function of donor HLA-B type were highly significant ($P = 0.0001$, Kruskal-Wallis test), whereas background reactivities of the other three control mAbs (Serotec, Immunotech, and Silenus) as a function of donor HLA-B type were similar (Fig. 1, other panels). Closer inspection of the One Lambda control mAb reactivities showed the lowest background reactivities with B22,n, B22,-, and B41,- lymphocytes, i.e., fewer than 2,000 MESF, which was with the same range as the reactivities of the other three control mAbs. The highest reactivities were observed with lymphocytes positive for HLA-B12, B13, B16, B17, B27, B37, and B47. A gene– dose effect (i.e., reactivity homozygous > heterozygous) was observed for HLA-B12, B13, B16, B17, and B27 (Fig. 1, lower left panel).

Anti–HLA-B27 mAb Test Results

As expected, the highest reactivities for all anti–HLA-B27 mAbs were observed against HLA-B27,- lymphocytes. For each anti–HLA-B27 mAb, cross-reactivities against various non–HLA-B27 antigens differed greatly and reached levels as high as those observed against HLA-B27,n lymphocytes. The reactivity patterns of the five anti–HLA-B27 mAbs tested are compared as a function of the donors' HLA-B types in Figure 2, in which test results of HLA-B heterozygous and homozygous donors are shown side by side.

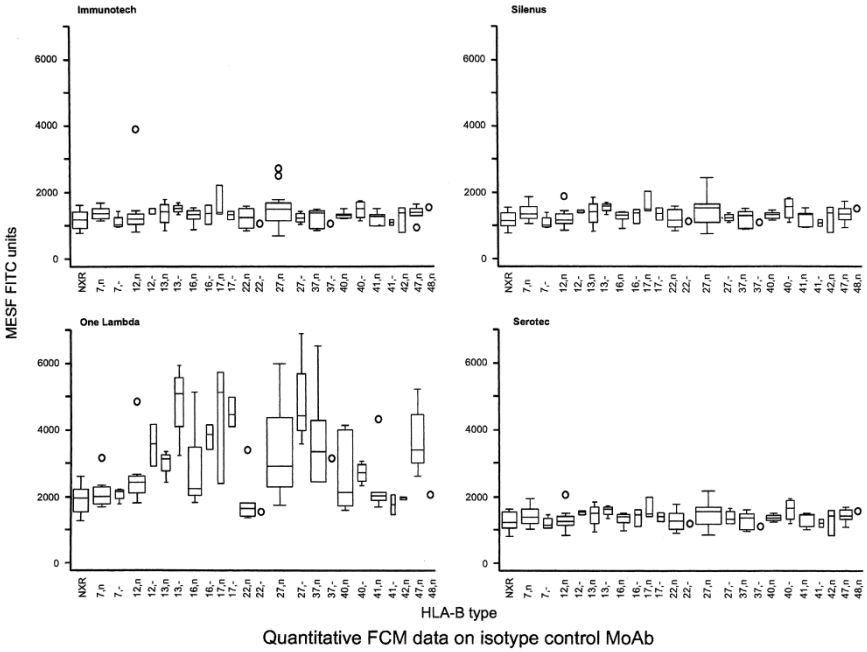

Figure 1: Reactivities of the fluorescein isothiocyanate (FITC)–labeled isotype control monoclonal antibody (MoAb) from Immunotech (upper left), Silenus (upper right), One Lambda (lower left), and Serotec (lower right) with the HLA-B heterozygous (designated HLA-Bx,n) and homozygous (designated HLA-Bx,-) panel donors. Fluorescence intensities are expressed as molecules of equivalent soluble fluorochrome (MESF) FITC. The width of the boxes are proportional to the numbers of observations within the respective groups. The line in the middle of each box represents the median of the data. The boxes extend from the 25th to the 75th centiles (i.e., the interquartile range). The lines rising from the boxes (i.e., the whiskers) extend to the upper and lower adjacent values, which are defined as 1.5 x the interquartile range rolled back to where there are data. Observed points more extreme than the adjacent values (i.e., outside values) are plotted individually (○). NXR, non–cross-reactive with HLA-B27.

HLA-B27 (Fig. 2, panels 1 and 2).

From the 12 HLA-B27 heterozygous donors, 10 were HLA-B*2705, one was HLA-B*2702, and one was HLA-B*2708. All three HLA-B27 homozygous donors were HLA-B*2705. Clone FD705 showed the weakest reactivity with HLA-B27+ lymphocytes and the strongest with clone GS145.2, whereas reactivities obtained with the three ABC-m3 reagents were between those values. This pattern was observed with heterozygous (P = 0.0001, Kruskal-Wallis test) and homozygous (P = 0.01) donors. Of note, lymphocytes of the single HLA-B*2708 donor were not recognized by the FD705 mAb.

Non–cross-reactive HLA-B alleles (Fig. 2, panel 3).

The non–cross-reactive HLA-B alleles were defined as any HLA-B allele other than B7, B12, B13, B16, B17, B22, B27, B37, B40, B47, and B48. The reactivities of the five mAbs differed significantly ($P = 0.0002$). The reactivities of clones FD705 and GS145.2 were lowest (median, 1,246 and 1,442 MESF, respectively), and those of the three ABC-m3 reagents ranged between 5,927 and 7,922 MESF, respectively.

HLA-B7 (Fig. 2, panels 4 and 5).

Clone FD705 stood out by the complete absence of cross-reactivity, even with lymphocytes of HLA-B7 homozygous donors (i.e., <2,000 MESF). In contrast, the Serotec ABC-m3 mAb showed strong cross-reactivity (heterozygous donors: median, 56,631 MESF; homozygous donors: median, 112,112 MESF). Moderate cross-reactivities were observed with the Immunotech ABC-m3 mAb and clone GS145.2, but the Silenus ABC-m3 mAb showed somewhat lower cross-reactivity. Analysis of lymphocytes from HLA-B7 homozygous donors showed a gene–dose effect in comparison with HLA-B7 heterozygous donors for all mAbs except FD705.

HLA-B12 (Fig. 2, panels 6 and 7).

Cross-reactivities of the three ABC-m3 and the FD705 mAbs were generally minor, although there was considerable heterogeneity in the intensity of cross-reactivities against the heterozygous donors. The cross-reactivity of the GS145.2 mAb was lowest (i.e., <2,000 MESF on all but two donors).

HLA-B13 (Fig. 2, panels 8 and 9).

All five mAb showed minor cross-reactivities with HLA-B13 heterozygous donors. Moderate cross-reactivities were observed
for the three ABC-m3 mAb with HLA-B13 homozygous donors, whereas minor cross-reactivities of GS145.2 and FD705 with these donors were noted.

HLA-B16 (Fig. 2, panels 10 and 11).

All three ABC-m3 and the FD705 mAbs showed minor cross-reactivities with HLA-B16 heterozygous donors and somewhat higher cross-reactivities with HLA-B16 homozygous donors. The cross-reactivity of the GS145.2 mAb was of moderate intensity with HLA-B16 heterozygous and homozygous donors. The intensity of cross-reactivity of the FD705 mAb with HLA-B16 heterozygous and homozygous donors varied greatly. Of note, all five mAbs cross-reacted strongly with one HLA-B16 homozygous donor for unknown reasons.

HLA-B17 (Fig. 2, panels 12 and 13).

The five mAb showed little, if any, cross-reactivity with lymphocytes from HLA-B17 heterozygous donors. Evaluation against HLA-B17 homozygous donors confirmed that the GS145.2 mAb was the least cross-reactive, whereas FD705 was the most cross-reactive.

HLA-B22 (Fig. 2, panels 14 and 15).

Clones FD705 and GS145.2 did not cross-react with HLA-B22 heterozygous donors, whereas the three ABC-m3 reagents showed moderate cross-reactivities, with the Serotec reagent being the highest. The cross-reactivity patterns against the HLA-B22 homozygous donor were similar to, but of slightly higher intensities than, those observed against the HLA-B22 heterozygous donors.

HLA-B37 (Fig. 2, panels 16 and 17).

Minor to moderate cross-reactivities was observed for all five mAb, with the strongest for the GS145.2 clone. The crossreactivity patterns against the HLA-B37 homozygous donor were similar to, but of slightly higher intensities than, as those observed against the HLA-B37 heterozygous donors.

HLA-B40 (Fig. 2, panels 18 and 19).

Minor and moderate cross-reactivities were observed for the three ABC-m3 mAbs against HLA-B40 heterozygous and homozygous donors, respectively. The cross-reactivities of the GS145.2 and FD705 mAbs were only minor, with those observed with the FD705 mAb being the lowest.

HLA-B41 (Fig. 2, panels 20 and 21).

Minor crossreactivities without the gene–dose effect were observed for the three ABC-m3 mAbs against HLA-B41 heterozygous and homozygous donors. Except for a few HLA-B41 heterozygous donors, clones GS145.2 and FD705 did not cross-react with HLA-B41.

HLA-B42 (Fig. 2, panel 22).

All three ABC-m3 and the GS145.2 mAbs showed moderate to strong cross-reactivities against HLA-B42 heterozygous donors, whereas the FD705 mAb was minimally cross-reactive, if at all.

HLA-B47 (Fig. 2, panel 23).

The three ABC-m3 and the GS145.2 mAbs showed minor cross-reactivities against HLA-B47 heterozygous donors. Clone FD705 showed moderate cross-reactivity, with large variations across donors.

HLA-B48 (Fig. 2, panel 24).

The three ABC-m3 and the FD705 mAbs showed weak cross-reactivities with the single HLA-B48 heterogenous donor, whereas clone GS145.2 showed moderate cross-reactivity.

Cumulative Cross-Reactivity Analysis

We used cross-reactivity scores (see Materials and Methods) to summarize the cross-reactivities of the five anti–HLA-B27 mAbs (Table 2). The least cross-reactivity was observed for FD705 (heterozygous donors, score of 11; homozygous donors, score of 15), followed by GS145.2 (heterozygous donors, score of 25; homozygous donors, score of 27). GS145.2 followed FD705 because of its cross-reactivity with the frequently occurring alleles HLA-B7 and B40, which could not be compensated for by its better performance with respect to cross-reactivity with HLA-B12. The three ABC-m3 mAbs performed less well. Among these reagents, the Silenus mAb scored best (heterozygous donors, score of 26; homozygous donors, score of 32) because it showed only minor cross-reactivity with the frequently occurring allele HLA-B7. The Immunotech mAb scored slightly worse (heterozygous donors, score of 32; homozygous donors, score of 33) due to its moderate cross-reactivity with HLA-B7. The Serotec mAb performed worst (heterozygous donors, score of 47; homozygous donors, score of 55), mainly due to its strong cross-reactivity with HLA-B7.

Evaluation of the Correctness of HLA-B27- Assignments

We used conclusion scores (see Materials and Methods) to summarize the failure of the Silenus ABC-m3 mAb, the HLA-B27 Screening Kit, and the FD705 mAb to generate appropriate negative conclusions on HLA-B27–negative donors expressing HLA-B antigens with documented serologic cross-reactivities with HLA-B27mAb (Table 3). Among the three ABC-m3 mAbs, we restricted this analysis to the Silenus mAb because it showed the lowest cross-reactivity (Table 2). When lymphocytes of heterozygous donors were tested, clone GS145.2 performed best (score of 2), failing to reach the negative conclusion on 13% of HLA-B7+ donors and 17% of HLA-B16+ donors. However, all four HLA-B7 and one of three HLA-B16 homozygous donors were assigned the FTR conclusion. The FD705 mAb performed best when homozygous donors were tested (score of 8), with 100% negative conclusions on the frequently occurring allele B7. However, this mAb performed slightly less well on HLA-B heterozygous donors,

Table 3: Conclusions obtained by single anti–HLA-B27 or combinations of two anti–HLA-B27 monoclonal antibodies[a]

	FTR (single mAb)			ABC-m3 & GS145.2		ABC-m3 & FD705		GS145.2 & FD705	
HLA-B allele (n)	ABC-m3[b]	GS-145.2	FD-705	FTR	POS	FTR	POS	FTR	POS
Heterozygous donors									
NXR (7)	0	0	0	0	0	0	0	0	0
B7 (8)	38	13	0	50	0	38	0	13	0
B12 (8)	13	0	13	13	0	25	0	13	0
B13 (4)	25	0	25	25	0	50	0	25	0
B16 (6)	0	17	17	17	0	17	0	33	0
B17 (3)	33	0	33	33	0	67	0	33	0
B22 (7)	86	0	0	86	0	86	0	0	0
B37 (7)	29	0	14	29	0	43	0	14	0
B40 (7)	0	0	0	0	0	0	0	0	0
B41 (7)	0	0	0	0	0	0	0	0	0
B42 (3)	67	0	0	67	0	67	0	0	0
B47 (6)	0	0	33	0	0	33	0	33	0
B48 (1)	0	0	0	0	0	0	0	0	0
Conclusion score[c]	9	2	5	11		14		7	
Homozygous donors									
B7 (4)	75	100	0	25	0	75	0	100	0
B12 (3)	0	0	0	0	0	0	0	0	0
B13 (4)	0	0	0	0	0	0	0	0	0
B16 (3)	33	33	67	0	33	33	33	33	33
B17 (3)	0	0	33	0	0	33	0	33	0
B22 (1)	100	0	0	100	0	100	0	0	0
B37 (1)	0	0	100	0	0	100	0	100	0
B40 (4)	50	0	25	50	0	25	25	25	0
B41 (2)	0	0	0	0	0	0	0	0	0
Conclusion score[c]	14	14	8	28		22		22	

Table 3: [a] Results shown are percentages of the number of donors tested for each (group of) HLA-B alleles. FTR, further typing required; POS, HLA-B27+; mAb, monoclonal antibody; NXR, non–cross-reactive HLA-B antigens (i.e., any HLA-B allele other than HLA-B27 and those listed in the table).

[b] Silenus ABC-m3 mAb.

[c] For computation of conclusion scores, see Materials & Methods.

with FTR conclusions on 13% to 33% of donors expressing HLA-B12 or the less frequent antigens B13, B17, B37, and B47. The least satisfactory results were obtained with the Silenus ABC-m3 mAb on HLA-B heterozygous and homozygous donors. That result was due only due to its cross-reactivity with HLA-B7 but also to its crossreactivities with several, less frequently occurring HLA-B antigens (Table 3).

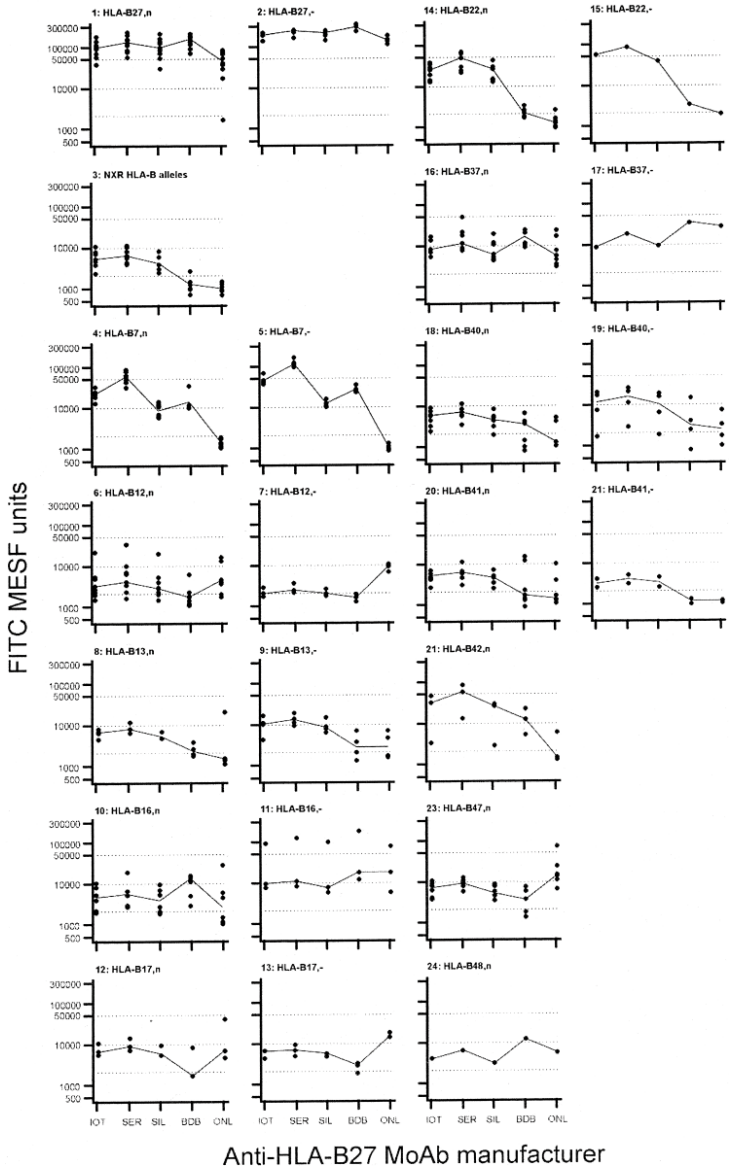

Figure 2: Reactivities of the five anti-HLA-B27 monoclonal antibodies (MoAb) with the HLA-B heterozygous and homozygous panel donors. For each reagent and HLA-B type, the median results have been connected with a line to highlight differences between antibodies for each HLA-B type. Logarithmic scales have been used for the y axes to compress the figure. Dotted lines have been drawn at the 2,000, 10,000, and 50,000 levels of molecules of equivalent soluble fluorochrome (MESF) as references to distinguish between absent, minor, moderate, and strong cross-reactivities (see Materials and Methods). BDB, Becton-Dickinson Biosciences; IOT, Immunotech; NXR, non–crossreactive; ONL, One Lambda; SER, Serotec; SIL, Silenus.

According to the EFI and ASHI guidelines, combinations of two different anti–HLA-B27 clones should be used to optimally control for cross-reactivity (22,23). Therefore, we evaluated the combined results obtained with the Silenus ABC-m3 mAb, the GS145.2 mAb kit, and the FD705 mAb (Table 3). No false positive conclusions were observed when any of the HLA-B heterozygous donors were tested, whereas the frequency of the FTR conclusion differed as a function of cross-reacting the HLA-B antigen and mAb combination. When HLA-B homozygous donors were tested, false positive conclusions were assigned to some donors expressing HLA-B16 or B40, depending on the mAb combination. Overall, the combination of the HLA-B27 Screening Kit and the FD705 mAb performed best, with the lowest scores for HLA-B heterozygous (i.e., 7) and homozygous (i.e., 22) donors.

DISCUSSION

To optimally advise the participants of the Benelux external quality assurance program for flow cytometric HLA-B27 screening, we performed a comprehensive analysis of the cross-reactivity patterns of the five FITC-labeled anti–HLA-B27 mAbs that were commercially available for this purpose in 2001. The reagents tested were derived from three mAb clones, i.e., ABC-m3 (Serotec, Immunotech, and Silenus), GS145.2 (BD Biosciences), and FD705 (One Lambda). The Serotec reagent contained FITC-labeled ABC-m3 only, whereas the Immunotech reagent also contained a PE-labeled anti–HLA-B7 mAb, and the Silenus reagent was formulated by using FITC-labeled anti–ABC-m3 and an unlabeled anti–HLA-B7 mAb. We used lymphocytes from a panel of donors selected to include HLA-B antigens with known cross-reactivities with HLA-B27 to optimally document any cross-reactivities of the studied reagents for flow cytometric HLA-B27 screening.

For the ABC-m3-based reagents, we observed strong cross-reactivities with HLA-B27- lymphocytes expressing HLA-B7, B22, B37, and B42, whereas weak cross-reactivities were observed with HLA-B12, B13, B16, B17, B40, B41, B47, and B48. These results confirmed and extended previous observations, specifically with respect to HLA-B7 (13,17–19,24–28), B12, and B37 (17,18). By using a scoring system to summarize the cross-reactivity patterns observed with the cell panel, we observed that the Serotec reagent showed clearly more cross-reactivity than the Immunotech or Silenus reagents. This result was due to the strong cross-reactivity of the ABC-m3 mAb with HLA-B7 in the Serotec reagent, whereas this cross-reactivity was reduced in the Immunotech and Silenus formulations containing anti-HLA-B7 mAb, presumably by blocking the cross-reactive sites.

For the GS145.2 mAb, we observed strong cross-reactivities with HLA-B7, B16, B37, B42, and B48 and weak crossreactivities with HLA-B13, B17, B22, B40, and B47. These results confirmed and extended previous observations, specifically with respect to HLA-B7 (13,16,17,19,29), B16, and B37 (13,16,17). We did not observe the previously reported cross-reactivity with HLA-B12 (17). This discrepancy may be explained by the possibility that the HLA-B12+ test cells in the previous study (17) coexpressed HLA-B antigens with hitherto unrecognized cross-reactivities with HLA-B27. By and large, the cross-reactivity score of the GS145.2 mAb was slightly better than that of the best performing ABC-m3–based reagent, i.e., from Silenus (scores 25 and 27 vs. scores 26 and 32 on lymphocytes from HLA-B heterozygous and homozygous donors, respectively).

For the FD705 mAb, we observed strong cross-reactivities with HLA-B17, B37, and B47 and weak cross-reactivities with HLA-B12, B13, B16, and B48. These results confirmed and extended those of previous studies, specifically with respect to the absence of HLA-B7 cross-reactivity (8,19,24) and the presence of cross-reactivities with HLA-B12, B17, and B37. Of note, the FD705 mAb was initially considered to be monospecific for HLA-B27 (8,19,24). Two of these studies (8,19) concluded that FD705 does not cross-react with antigens belonging to the HLA-B27 CREG, but certainly not all HLA-B antigens had been tested. The third study (24) evaluated the FD705 mAb against only a small panel (i.e., 10) of donors that did not include, e.g., HLA-B16, B17, and B37. On the basis of the combined results of our and other studies, as discussed above, we concluded that FD705 is not monospecific for HLA-B27 but does show the lowest degree of cross-reactivity with other HLA-B antigens in comparison with the ABC-m3 and GS145.2 mAbs.

In 2002, the ABC-m3–based Com-B27 cocktail was launched as a reagent for flow cytometric HLA-B27 screening by IQ Products (Groningen, The Netherlands). This reagent was recently compared with the FD705 mAb (31). A preliminary analysis showed that the Com-B27 cocktail cross-reacted with HLA-B7 but not with HLA-B44 (B12). A minor advantage of the Com-B27 cocktail over FD705 was that it bound to the protein encoded by the B*2708 allele, which is unrecognized by the FD705 mAb (31, present study).

We used quantitative flow cytometry to express the intensity of cross-reactivities of flow cytometric HLA-B27 screening reagents with other HLA-B antigens. The quantitation of experimental results as MESF FITC, an instrument-independent parameter of fluorescence intensity, allowed the meaningful comparison of results over time with one instrument (relevant for our study) and between instruments (32). Thus, we could compare our results with those of a previous study using the same approach (33). Indeed, the fluorescence intensity observed with the FITC-labeled FD705 mAb

on HLA-B27+ and HLA-B27- lymphocytes were within the same orders of magnitude in both studies.

The ASHI and EFI standards for histocompatibility testing require that flow cytometric HLA typing be set up so that "a single monoclonal antibody may be used to define an antigen provided its monospecificity has been sufficiently verified by local testing" (22,23). It is safe to conclude that neither the five HLA-B27 screening reagents tested in this study nor the newer Com-B27 cocktail (33) fulfill this criterion. Therefore, verification of HLA-B27 positivity by an independent technique is required in case of a positive result obtained by flow cytometry. When one reagent had been used for HLA-B27 screening of our HLA-B27- panel, the ABC-m3–based reagents would have required more verifications and the GS145.2-based screening kit would have required the fewest. The fact that the GS145.2-based screening kit would have required less retesting than the less cross-reactive FD705 mAb is due to the different analytical strategies employed. The GS145.2-based screening kit comes with software in which the cutoff between positive and negative already corrects for the relatively dim fluorescence intensity associated with most cross-reactivities of the GS145.2 mAb. In contrast, One Lambda recommends the use of an isotype control mAb with the FD705 mAb. Although the isotype control mAb provided by One Lambda has a relatively high level of background reactivity depending on the HLA-B type of the cell donor, this threshold is less stringent in excluding positivity due to cross-reactivity than the approach used by the GS145.2-based screening kit.

The use of two HLA-B27 screening reagents to obtain a definite HLA-B27 typing result by flow cytometry on our cell panel would have avoided any false-positive conclusion on the HLA-B27-, HLA-B heterozygous donors. On HLA-B27-, HLA-B homozygous donors, any combination of ABC-m3 (Silenus), GS145.2, and FD705 would have classified one HLA-B16 homozygous donor as HLA-B27+, whereas the combination of ABC-m3 (Silenus) and FD-705 would have classified a second HLA-B40 homozygous donor as HLA-B27+. This analysis demonstrated that even this stringent and relatively costly strategy (many laboratories do not use two HLA-B27 screening reagents for flow cytometric HLA-B27 typing because of the high cost) (13) is not absolutely error proof. Conflicting results between any combination of two HLA-B27 screening reagents would require further testing by an independent technique. Based on our cell panel, further testing would have been reduced to a minimum by a combination of the FD705 mAb and the GS145.2-based screening kit on HLA-B27-, HLA-B heterozygous donors and by a combination of the FD705 mAb and the GS145.2-based screening kit or the Silenus ABC-m3 mAb on HLA-B27-, HLA-B homozygous donors. Based on these results, we recommend the combination of the FD705 mAb and the GS145.2-based screening kit as the most robust approach for flow cytometric HLA-B27 screening.

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Wilfried H.B.M. Levering
Kees Sintnicolaas
Henk Wind
Herbert Hooijkaas
Jan W. Gratama

FLOW CYTOMETRIC SCREENING FOR THE HLA-B27 ANTIGEN ON PERIPHERAL BLOOD LYMPHOCYTES

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INTRODUCTION

A strong association between HLA-B27 and ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system, was demonstrated in the early 1970s (Brewerton *et al*., 1973). Nowadays it is evident that HLA-B27 is common to the entire group of seronegative spondyloarthropathies. Of these forms of arthritis, the association with HLA-B27 is 90% for spondylitis ankylopoietica (Bechterew's disease), 75% for reactive arthritis (including Reiter's syndrome), 50% to 60% for arthritis psoriatica, and 60% to 90% for enteropathic arthritis (the latter two with spinal abnormalities). An eye inflammation, termed uveitis anterior acuta, is also associated with HLA-B27 and related to the seronegative spondyloarthropathies; about 50% of these patients express HLA-B27. These combined results demonstrate that assessment of HLA-B27 is of diagnostic importance if any of these diseases are considered. Therefore, screening for HLA-B27 is performed in many clinical diagnostic laboratories.

Traditionally, HLA typing is performed with the complement-dependent cytotoxicity (CDC) assay (Mittal *et al*., 1968), which is widely used in tissue-typing laboratories. However, the CDC assay is time consuming and expensive if used for HLA-B27 screening only, as it requires specialized knowledge and extensive quality-control procedures. Therefore, alternative methods have been developed for HLA-B27 screening: (i) flow cytometry (FCM), (ii) HLA-B27 genotyping by polymerase chain reaction (PCR) assays, and (iii) enzyme-linked immunosorbent assays. Among these alternative methods, flow cytometric HLA-B27 screening is most widely used in general diagnostic laboratories, as it is simple, rapid, and cost effective.

This unit describes a flow cytometric protocol to screen for HLA-B27 on peripheral blood lymphocytes. The Basic Protocol features the use of more than one anti-HLA-B27 monoclonal antibody (MAb) to detect possible cross-reactivity of these reagents with non-HLA-B27 antigens. According to the American Society for Histocompatibility and Immunogenetics (ASHI) Standards for Histocompatibility Testing issued in 2000 and the European Federation for Immunogenetics (EFI) Standards for Histocompatibility Testing version 5.3 (issued in 2003), the use of positive and negative control cells is incorporated. The approach to flow cytometric HLA-B27 screening described in this protocol can be used to study blood samples that have been anticoagulated using ethylenedinitrilotetraacetic acid (EDTA), heparin, or acid citrate dextrose solution formula A (ACD-A), the most commonly used anticoagulants in general diagnostic laboratories.

Flow cytometric HLA-B27 screening is hampered by the lack of true monospecific anti-HLA-B27 antibodies (monoclonal antibodies; MAbs), owing to the fact that HLA-B27 belongs to the large HLA-B7 cross-reacting group (CREG). Cross-reactivities of anti- HLA-B27 MAbs with other HLA-B antigens have been reported (Lingenfelter *et*

al., 1995; Neumüller *et al*., 1996; Hoffmann and Janssen, 1997; Coates and Darke, 1998; Levering *et al*., 2000, 2003). To discern the possibility of cross-reactions of anti-HLA-B27 MAbs with non-HLA-B27 antigens, it is important that the anti-HLA-B27 Mabs used have nonoverlapping cross-reactivities. Nowadays, three MAb clones are commonly used in clinical laboratories: ABC-m3, GS145.2, and FD705. ABC-m3 is commercially available from Serotec, Immunotech, and Silenus Laboratories. GS145.2 is provided by Becton Dickinson (BD) Biosciences and FD705 by One Lambda (*http://www.onelambda.com*). The authors have performed a systematic study of crossreactivities of these five MAbs against HLA-B27 by using a panel of cell donors expressing the range of known HLA-B antigens that have documented cross-reactivities with HLA-B27 (Levering *et al*., 2003), and recommend the combined use of GS145.2 and FD705. This combination avoids most false-positive conclusions, especially on HLA-B27^{neg}, HLA-B heterozygous samples.

For data acquisition, a multiparameter flow cytometry methodology is applied. Information from three to four parameters—i.e., forward scatter (FS), side scatter (SS), HLA-B27 expression, and, in some cases CD3 expression or B7 expression—is acquired. Lyse-and-wash sample processing is employed, suitable for all the aforementioned MAbs.

MATERIALS AND METHODS

Materials

1. Patient blood samples to be screened for HLA-B27, anticoagulated with acid citrate dextrose or EDTA
2. Positive control: freshly obtained blood sample with known HLA-B27-positive HLA-B typing result
3. Negative control: freshly obtained blood sample with known HLA-B27-negative HLA-B typing result and no cross-reactivity with the HLA-B27 MAb used (see Commentary)
4. HLA-B27 Kit (BD Biosciences; optional) containing:
   - Anti-HLA-B27-FITC/CD3-PE (clones anti-GS145.2 and SK7, respectively)
   - 10× BD FACS lysing solution
   - Calibration beads
   - HLA-B27 Kit software (optional)
5. 10× ammonium chloride lysing solution or BD FACS lysing solution (BD Biosciences)
6. Phosphate-buffered saline (PBS) containing 0.2% BSA
7. PBS containing 1% (w/v) paraformaldehyde

8. FITC-conjugated anti-HLA-B27 monoclonal antibodies: GS145.2 (BD Biosciences), and FD705 (One Lambda; *http://www.onelambda.com*); combined use of GS145.2 and FD705 is recommended (see above)
9 FITC-conjugated mouse Ig2b isotype control MAb (One Lambda, cat. no. G2B-F50X)
10. 12 × 75–mm polypropylene tubes
11. Tabletop centrifuge
12. Flow cytometer equipped to detect forward scatter (FS) and side scatter (SS), with a laser emitting at 488 nm and at least two fluorescence detectors, i.e., for fluorescein isothiocyanate (FITC) and phycoerythrin (PE)

Prepare samples

For FD705 MAb

1a. Pipet 100 µl blood sample into tubes 1 to 4 (see Table 6.22.1). Include as controls a freshly obtained blood sample with known HLA-B27-positive typing result and a freshly obtained blood sample with known HLA-B27-negative typing result, without cross-reactivity with the HLA-B27 MAb to be used.
2a. Add the amounts of MAb recommended by the manufacturer to tubes 1 to 4 (see Table 6.22.1). Gently mix by vortexing.

Table 6.22.1: Staining protocol for flow cytometric HLA-B27 staining

Staining no.	Specimen	Reagents
1	Patient specimen	IgG2b-FITC
2	Patient specimen	FD705-FITC
3	Positive control	FD705-FITC
4	Negative control	FD705-FITC
5	Patient specimen	GS145,2-FITC/CD3-PE
6	Positive control	GS145,2-FITC/CD3-PE
7	Negative control	GS145,2-FITC/CD3-PE
8	HLA-B27 calibration beads	----

For GS145.2 MAb (BD Biosciences HLA-B27 kit)

1b. GS145.2 MAb (BD Biosciences HLA-B27 kit): Pipet 50 µl blood sample into tubes 5 to 7 (see Table 6.22.1). Include the same positive and negative controls as in step 1a.

Prepare an additional tube (tube 8) with HLA-B27 calibration beads alone according to the manufacturer's instructions.

All specimens for HLA-B27 screening should be tested within 24 hr of collection. Clearly deteriorated samples, such as those with large clots or significant hemolysis, are unacceptable and must be discarded.

CAUTION: All cell suspensions must be considered infectious unless fixed in medium containing 1% paraformaldehyde. The MAbs and calibration standards are supplied in NaN3-containing media in most cases; waste fluids containing NaN3 must be discarded according to institutional guidelines.

Currently, no long-term stabilized HLA-B27 typed control samples are available for this assay. Instead, cryopreserved and thawed mononuclear cell suspensions with known HLA-B27 typing results may be used, provided that the thawed cells show reactivity patterns with the HLA-B27 screening reagents (i.e., fluorescence intensity levels) similar to those of the same cells when tested freshly (Coates and Darke, 1998). This validation must be done by each individual laboratory.

IMPORTANT NOTE: The calibration bead suspension must be prepared immediately prior to calibration (see step 9b).

2b. Add the amount of anti-HLA-B27-FITC/CD3-PE MAb (provided with kit) as recommended by the manufacturer to tubes 5 to 7 (see Table 6.22.1). Gently mix by vortexing.

3. Incubate tubes 15 min at room temperature (20º to 25ºC) in the dark.

For FD705 MAb

4a. Prepare 1× working lysis solution by diluting either 10× ammonium chloride lysing solution or BD FACS 10× lysing solution (provided with the kit) 1:10 with distilled water. Add 2 ml of 1× ammonium chloride lysing solution or 1× BD FACS lysing solution to tubes 1 to 4. Gently mix by vortexing. Incubate the tubes 10 min at room temperature in the dark.

The working solution of 1× ammonium chloride lyse should be kept cold and the unused portion discarded at the end of the day. The BD FACS lysing solution (BD Biosciences) is stable for 1 month when stored in a glass container at room temperature (20º to 25ºC).

5a. Centrifuge tubes 1 to 4 for 5 min at 400 × g, room temperature.

6a. Decant the supernatants and resuspend each cell pellet in 2 ml PBS containing 0.2% BSA. Centrifuge tubes 1 to 4 for 5 min at 400 × g, room temperature. Remove supernatants, then repeat this step for a second wash with PBS/0.2% BSA. After the second wash, aspirate the supernatants without disturbing the pellets.

GS145.2 MAb (BD Biosciences HLA-B27 kit)

4b. Prepare 1× working lysing solution by diluting the 10× BD FACS lysing solution (provided with the kit) 1:10 with distilled water. Add 2ml of the 1×BD FACS lysing solution to tubes 5 to 7. Gently mix by vortexing. Incubate the tubes 10 min at room temperature in the dark.

5b. Centrifuge tubes 5 to 7 for 5 min at 300 × g, room temperature.

6b. Decant the supernatants and resuspend each cell pellet in 2 ml PBS containing 0.2% BSA. Centrifuge tubes 5 to 7 for 5 min at 200 × g, room temperature. Remove supernatants, then repeat this step for a second wash with PBS/0.2% BSA. After the second wash, aspirate the supernatants without disturbing the pellets.

7. Add 0.25 ml PBS containing 1% paraformaldehyde to all tubes (tubes 1 to 7) and gently mix by vortexing.

8. Perform flow cytometric data acquisition as described in the remaining steps on the same day. Store samples at 2º to 8ºC, protected from light, if not to be analysed immediately.

Set up instrument and gating regions

9a. *For FD705 MAb*: Set up instrument to collect linear forward scatter (FS), linear side scatter (SS), log green fluorescence (FITC), and log orange fluorescence (PE) according to the manufacturer's instructions.

9b. *For GS145.2 MAb (BD Biosciences HLA-B27 kit)*: Vortex the calibration bead vial (provided with the kit) gently but thoroughly. Add 2 drops of beads to 1 ml flow diluent in a tube. Vortex the bead suspension at low speed for 3 sec. If using a BD Biosciences flow cytometer, calibrate log green fluorescence (FITC) and linear forward scatter (FS) by running the calibration beads, utilizing the HLA-B27 software. For Beckman Coulter instruments, or when HLA-B27 software is not used, set up the green fluorescence window of analysis by adjusting the PMT-voltage until the median fluorescence intensity (MFI) of the calibration beads matches the bead suffix provided with the beads. The bead suffix, printed on the vial with calibration beads, represents the channel number matching with the median fluorescence intensity (MFI) of the calibration beads.

In the following steps, specific terminology for regions and gates are given parenthetically for different instruments. Region A is for Beckman Coulter instruments, and region R1 and gate G1 are for BD Biosciences instruments.

Regarding the GS145.2 MAb, users of BD Biosciences FACS series instrument should use the dedicated HLA-B27 software for instrument set up, gating regions, data acquisition, and data analysis according to the manufacturers' instructions.

IMPORTANT NOTE: The bead suffix is based on a 256-channel resolution (linear histogram channels). If the channel resolution is 1024, the bead and reagent suffix should be multiplied by a factor of 4.

10. Create the following histograms:
    - Histogram 1: FS versus SS
    - Histogram 2: green fluorescence versus cell count
    - Histogram 3: FS versus orange fluorescence.
11. In histogram 1, display all events. Draw a rectangular gate (A; R1) to include all lymphocytes (lymphocyte gate).
12. In histogram 2, display events from the lymphocyte gate (i.e., gated on A; gate G1 = R1).
13. In histogram 3, display all events. Draw a rectangular gate (B; R2) to include at least 50% of the CD3+ lymphocytes, (CD3+ gate).
14. In histogram 2, display events from the CD3+ gate (i.e., gated on B; gate G2 = R2).

Acquire and analyze data

For the FD705 MAb

15a. Acquire 15,000 ungated events from each tube. At least 3,000 lymphocyte events (see step 16) should be included.

16a. First run the IgG1-FITC isotype control. Select the lymphocytes on the basis of their light-scatter characteristics (i.e., FS low, SS low).

17a. Print histogram 2 of the selected events. Set a marker to discriminate between positive and negative fluorescence as close as possible to the right-hand foot of the negative population, in such a way that the percentage of positive events is 2% of the gated events.

18a. Run tubes 2 to 4. Select the lymphocytes using the same gating region as for the control.

19a. Perform histogram statistics for histogram 2 using the marker set on the control in step 17a.

Samples with a marked (i.e., a substantial or noticeable) shift relative to the marker are considered to be positive for HLA-B27.

To acquire and analyze data for GS145.2 MAb (Beckman Coulter instruments)
Users of BD Biosciences FACS series instrument should use the dedicated software provided with the HLA-B27 for instrument set up, setting up of gating regions, data acquisition, and data analysis according to the manufacturers' instructions.

15b. Acquire a minimum of 2,000 CD3+ events (i.e., FS low, PE+) from each tube (5 to 7). Display the CD3+ events in the green fluorescence histogram and determine the MFI of the HLA-B27 FITC signal.

16b. For Beckman Coulter instruments, or when HLA-B27 software is not used, compare the MFI of the HLA-B27 FITC signal with the predefined decision marker.

The decision marker is encoded in the suffix of the reagent lot number listed on the vial label. This reagent suffix number represent the channel number matching the decision marker. Samples with a green fluorescence MFI result greater than or equal to the decision marker should be considered HLA-B27 positive. Samples with a green fluorescence MFI result lower than the decision marker should be considered HLA-B27 negative.
IMPORTANT NOTE: The reagent suffix is based on a 256-channel resolution (linear histogram channels). If the channel resolution is 1024, the bead and reagent suffix should be multiplied by a factor of 4.

Reagents and solutions

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A of CPC; for suppliers, see SUPPLIERS APPENDIX of CPC.

1. Phosphate buffered saline (PBS) containing 0.2% BSA.
   - Prepare/obtain the following stock solutions: Phosphate-buffered saline (PBS; APPENDIX 2A), pH 7.8
   - 30% (w/v) bovine serum albumin (BSA), immunohematology grade (e.g., Serologicals Corp.; http://www.serologicals.com)
   - 10% (w/v) NaN3 solution
   - 1 N HCl or 1 N NaOH

   Add 7 ml of 30% BSA solution to 983 ml PBS. Check pH and adjust to 7.7 to 7.8 by addition of either 1 N HCl or 1 N NaOH. Add 10 ml of 10% NaN3 solution. Store up to 2 weeks at 2º to 8ºC.

   CAUTION: NaN3-containing media are toxic and must be disposed of according to institutional safety guidelines.

COMMENTARY

Background Information

Traditionally, HLA typing has been performed using the complement-dependent cytotoxicity (CDC) assay, which is time consuming and expensive, requiring specialized knowledge and extensive quality-control procedures. Therefore, flow cytometric HLA-B27 screening is most widely used in clinical diagnostic laboratories. Currently, three different anti-HLA-B27 MAb clones are commercially available. Clone ABC-m3 (e.g., from Serotec, Immunotech, and Silenus Laboratories) was the first commercially available MAb for flow cytometric HLA-B27 screening. Unfortunately, cross-reactivity with non-HLA-B27 antigens was observed, especially with HLA-B7, a very common HLA-B antigen in Caucasians (Table 6.22.2). Later on, other clones, e.g., FD705 (One Lambda) and GS145.2 (BD Biosciences), became available. However, these anti-HLA-B27 Mabs showed more or less similar cross-reactivity patterns. The performance of MAb cocktails (i.e., the addition of unlabeled HLA-B7 MAb to block HLA-B7 antigens), compared with that of a single anti-HLA-B27 MAb, shows only small improvement. More specific details can be found in Critical Parameters and Troubleshooting. When appropriately performed and interpreted, flow cytometry can produce high-volume, cost-effective, and rapid turnaround of selected clinical tests. However, DNA-based typing methods, which nowadays constitute a gold, but costly, standard, are already commercially available for HLA-B27 typing. As molecular biology applications become more common in clinical diagnostic laboratories in the near future, the authors predict that flow cytometric HLA-B27 screening will lose some ground to molecular typing.

Critical Parameters and Troubleshooting

Cross-reactivity of anti-HLA-B27 MAb with other HLA-B alleles.

HLA-B27 belongs to the large HLA-B7 cross-reacting group (CREG). Within the HLA-B7 CREG, HLA-B13, B22, B27, B40, B41, B42, B47, and B48 share common epitopes with HLA-B7 (Fuller *et al*., 1990). In addition, cross-reactivities of HLA-B27 typing reagents with non HLA-B7 CREG antigens, i.e., HLA-B12, B16, B17, B37, and B73, have been reported (Lingenfelter *et al*., 1995; Hoffmann and Janssen, 1997; Coates and Darke, 1998; Levering *et al*., 2000, 2003). The cross-reactivities of human sera used in CDC assays are well known. However, murine monoclonal antibodies against HLA-B27 cross-react as well. Nowadays, the MAbs used include ABC-m3 (e.g., available through Serotec, Immunotech, and Silenus Laboratories), GS145.2 (BD Biosciences), and FD705 (One Lambda). Many studies have been published regarding the cross-reactivities of anti-HLA-B27 MAbs. The combined analysis of the results of the studies summarized in Table 6.22.2 identifies HLA-B7 as the major cross-reacting antigen

for two of the three abovementioned MAb clones, followed by HLA-B12 and HLA-B37 for all MAb clones. However, the combined data of these studies showed inconsistent patterns with respect to the cross-reactivities of anti-HLA-B27 MAbs with other HLA-B antigens, e.g., HLA-B13, HLA-B17, HLAB22, HLA-B39(16), HLA-B41, HLA-B42, and HLA-B73.

Table 6.22.2: Cross-reactivities of anti-HLA-B27 MAb: summary of published studies[a]

Cross-reactivity with HLA-B antigens-	Anti-HLA-B27 MAb		
	ABC-m3	GS145.2	FD705
B7	1,3-5,6,7-11	1,2,3,5,12	3
B12	1,3,4	1,3	1,3
B13	10		
B17		1,3	
B22	4,9,10	2,3	
B37	1,3,4	1,2,3	1,3
B39(16)	1,2		
B41		2	
B42	4,6		
B73	1,4,6		
None		13	5,7,14

Table 6.22.2: [a] Numbers refer to referenced publications: 1 = Levering *et al*. (2000); 2 = Lingenfelter *et al*. (1995); 3 = Hoffmann and Janssen (1997); 4 = Coates and Darke (1998); 5 = Levering *et al*. (2003); 6 = Coates *et al*. (2003); 7 = Orr *et al*. (1994); 8 = Trapani *et al*. (1983); 9 = Albrecht and Muller (1987); 10 = Kravtzoff *et al*. (1991); 11 = Reynolds *et al*. (1996); 12 = Hulstaert *et al*. (1994); 13 = Chou *et al*. (1997); 14 = Ward *et al*. (1995).

Choice of anti-HLA-B27 MAb.

A systematic study of cross-reactivities of the anti-HLA-B27 MAbs ABC-m3, GS145.2, and FD705 has been performed, using a panel of cell donors selected to express the range of all known HLA-B antigens with documented serological cross-reactivities with HLA-B27 (Levering *et al*., 2003).

ABC-m3-based reagents cross-react strongly with HLA-B27 negative lymphocytes expressing HLA-B7, -B22, -B37, and -B42, and cross-react weakly with HLA-B12, -B13, -B16, -B17, -B40, -B41, -B47, and -B48. These results confirm and extend previous observations, specifically with respect to HLA-B7, -B12, and -B37 (Table 6.22.2). The Serotec reagent clearly showed more cross-reactivity than the Immunotech or Silenus reagents. This result was due to the strong cross-reactivity of the ABC-m3 MAb with HLA-B7 in the Serotec reagent, while this cross-reactivity was reduced in the Immunotech and Silenus formulations containing anti-HLA-B7 MAb, presumably by blocking

of the cross-reactive sites. Another ABC-m3-based reagent, Com-B27 (Chemicon), cross-reacts with HLA-B7, -B42, and -B73 (Coates *et al*., 2003).

For the GS145.2 MAb, strong crossreactivities with HLA-B7, -B16, -B37, -B42, and -B48 and weak cross-reactivities with HLA-B13, -B17, -B22, -B40, and -B47 were observed. These results confirm and extend previous observations, specifically with respect to HLA-B7, -B16, and -B37 (Table 6.22.2). By and large, the GS145.2 MAb performs better than the ABC-m3-based reagents.

For the FD705 MAb, strong crossreactivities with HLA-B17, -B37, and -B47, and weak cross-reactivities with HLA-B12, -B13, -B16, and -B48 were observed. These results confirm and extend those of previous studies, specifically with respect to the absence of HLA-B7 cross-reactivity and the presence of cross-reactivities with HLA-B12, -B17, and -B37 (Table 6.22.2). Of note, the FD705 MAb was initially considered to be monospecific for HLA-B27 Olerup, 1994; Orr *et al*., 1994; Neumüller *et al*., 1996.

The Standards for Histocompatibility Testing of the ASHI (released in 2000) and the EFI (v. 5.3, released in 2003) permit the use of a single monoclonal antibody for flow cytometric HLA typing to define an antigen, provided that its monospecificity has been sufficiently verified. None of the abovementioned HLA-B27 screening reagents fulfils this criterion. Therefore, verification of HLA-B27 positivity by an independent technique is required in the case of a positive result obtained by flow cytometry. When only a single reagent is used for HLA-B27 screening, the ABC-m3-based reagents would require the most verifications and the GS145.2-based screening kit the fewest. The fact that the GS145.2-based screening kit requires less retesting than the less cross-reactive FD705 MAb is due to the different analytical strategies employed. The GS145.2-based screening kit comes with software in which the cutoff between "positive" and "negative" already corrects for the relatively dim fluorescence intensity associated with most cross-reactivities of the GS145.2 MAb. In contrast, the other companies recommend the use of an isotypecontrol MAb. In general, the threshold obtained with the isotype control is less stringent in excluding positivity due to cross-reactivity than the approach used by the GS145.2-based screening kit.

The use of two HLA-B27 screening reagents to obtain a definite HLA-B27 typing result by flow cytometry avoids, for the most part, a false-positive conclusion for the HLA-B27-negative, HLA-B heterozygous samples. For example, on HLA-B27-negative, HLA-B homozygous samples, any combination of ABC-m3 (Silenus), GS145.2, and FD705 would classify a single HLA-B16 homozygous donor as HLA-B27^{pos}, whereas the combination of ABC-m3 (Silenus) and FD705 would classify a second HLA-B40 homozygous donor as HLA-B27^{pos} (Levering *et al*., 2003).

This reveals that even this stringent and relatively costly strategy is not absolutely error proof. Conflicting results between any combination of two HLA-B27 screening reagents requires further testing by an independent technique (Table 6.22.3). Further

testing would be reduced to a minimum by a combination of the FD705 MAb and the GS145.2-based screening kit on HLA-B27-negative, HLA-B heterozygous samples, and by a combination of the FD705 MAb and the GS145.2-based screening kit or the Silenus ABCm3 MAb on HLA-B27-negative, HLA-B homozygous samples. Therefore, the combination of the FD705 MAb and the GS145.2-based screening kit is recommended

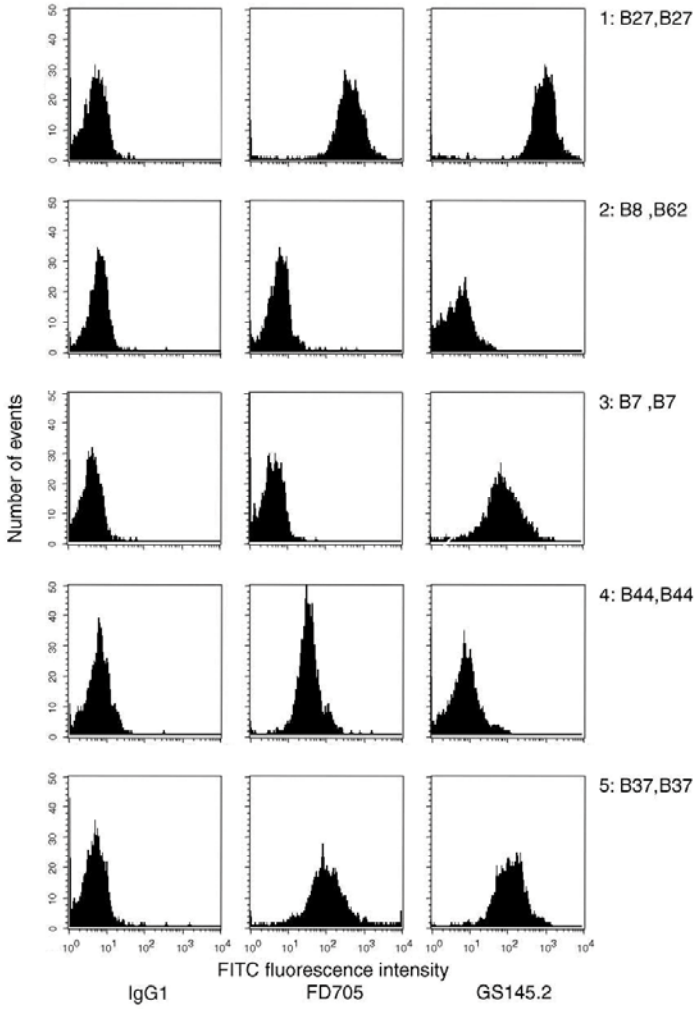

Figure 6.22.1: FITC fluorescence histograms obtained with the Basic Protocol for peripheral blood lymphocytes from five healthy donors. Left column, IgG1 isotype control MAb for the FD705 MAb (reactivity with lymphocytes); middle column, FD705 MAb (reactivity with lymphocytes); right column, GS145.2 MAb (reactivity with CD3+ lymphocytes). Each row represents a single donor; the serological HLA-B typing results are indicated on the right-hand side.

as the most robust approach for flow cytometric HLA-B27 screening. Examples of FITC fluorescence histograms obtained with this anti-HLA-B27 MAb combination on samples from five healthy donors are shown in Figure 6.22.1.

Table 6.22.3: Data interpretation of flow cytometric HLA-B27 screening

FD705 MAb result	GS145.2 MAb (HLA_B27 Kit) result	Conclusion
Negative	Negative	Negative
Negative	Positive	Further typing required[a]
Weakly positive	Negative	Negative
Weakly positive	Positive	Positive
Positive	Negative	Further typing required[a]
Positive	Positive	Positive

Table 6.22.3: [a] Full HLA-typing required (e.g., with serological, DNA-based techniques, or ELISA).

Reactivity of anti-HLA-B27 MAb with molecularly defined HLA-B27 subtypes.

An additional problem is that the reactivity of anti-HLA-B27 MAb with the different HLA-B27 alleles (as defined by DNA typing) may vary. Currently, 26 HLA-B27 alleles are known and have been assigned by the WHO Nomenclature Committee for Factors of the HLA System. An overview of published reactivities with HLA-B27 alleles is shown in Table 6.22.4.

Table 6.22.4: Reactivity of various HLA-B27 monoclonal antibodies with different HLA-B27 alleles[a]

	Anti-HLA-B27 MAb		
B27 allele	ABC-m3	GS145.2	FD705
B*2701	nt	nt	+ (1)
B*2702	+ (2,3)	+ (3)	weak (2,3); +(1)
B*2703	-/weak (3)	+ (3)	+ (1,3)
B*2704	nt	+ (4)	+ (1)
B*2705	+ (2,3,5)	+ (3,4)	+ (1,3,4,5)
B*2706	+ (3)	+ (3)	+ (1,3)
B*2707	nt	nt	+ (1)
B*2708	+ (2,5)	nt	- (2,5)
B*2709-2727	nt	nt	nt

Table 6.22.4: [a] nt = not tested. Numbers refer to referenced publications: 1 = Pei *et al*. (1993); 2 = Coates

Control cells.

For tissue-typing laboratories, HLA-B27-negative and -positive control cells must be included in routine flow cytometric HLA typing. Freshly obtained blood samples with known HLA-B27-positive and -negative HLA-B typing would be ideal, but logistically

very difficult to obtain. Currently, no suitable long-term stabilized control blood samples are available for this assay. Meanwhile, cryopreserved and thawed mononuclear cell suspensions may be used, provided that the thawed cells show reactivity patterns with the HLA-B27 screening reagents (i.e., fluorescence intensity levels) similar to those of the same cells when tested freshly (Coates and Darke, 1998).

Additional tests.

When flow cytometry results are inconclusive, further typing will be necessary to allow HLA-B27 assignment. Currently, the "gold standard" for HLA-B27 assignment is the complement-dependent cytotoxicity assay using a large panel of well-characterized human antisera (Mittal *et al*., 1968). Typically, this method is performed only by specialized laboratories. Alternative methods for HLA-B27 typing include (i) HLA-B27 genotyping by polymerase chain reaction (PCR) assays based on sequence-specific primers (PCR-SSP; Olerup, 1994), (ii) HLA-B27 genotyping by PCR assays based on sequence-specific oligonucleotides (PCR-SSO; Dominguez *et al*., 1992), (iii) HLA-B27 genotyping by fluorescent resonance emission transfer (FRET) probes in real-time PCR (Faner *et al*., 2004), (iv) HLA-B27 genotyping by SNP analysis using TaqMan technology (Behrens and Lange, 2004), (v) HLA-B27 genotyping by an allelespecific PCR melting assay (Seipp *et al*., 2005), and (vi) enzyme-linked immunosorbent assays (Chou *et al*., 2001). Again, these techniques require specialized knowledge and extensive quality-control procedures, and therefore are performed mainly by specialized tissue-typing laboratories.

Anticipated Results.

Samples with a fluorescence signal of intensity similar to that of the HLA-B27-positive control cells are unambiguously classified "strongly positive." Samples with a fluorescence signal exceeding that of the HLA-B27-negative control cells but less than the HLA-B27-positive control cells are classified "weakly positive." Samples with a fluorescence signal similar to that of the HLA-B27-negative control cells are classified "negative." Using MAb GS145.2 (provided with the BD Biosciences HLA-B27 kit), only the classifications "positive" and "negative" are applicable. The protocol for data interpretation is shown in Table 6.22.3.

Quantitative flow cytometry enables one to express the intensity of cross-reactivities of flow cytometric HLA-B27 screening reagents with other HLA-B antigens. The expression of experimental results as molecules of equivalent soluble fluorochrome (MESF), an instrument-independent parameter of fluorescence intensity, allows the quantitative comparison of results over time within a single instrument and between instruments (Schwartz *et al*., 1996). While this approach may not be needed for the interpretation of HLA-B27 screening results in everyday flow cytometry practice, it is

useful to perform quality control of the instrument's performance as regards measurement of fluorescence intensity.

Time Considerations.

Preparation of an individual sample or a small series of up to 10 samples takes 40 to 50 min, including washing steps. Assuming that the flow cytometer has been calibrated and set up and that the customer-defined software applications have been prepared, listmode data acquisition takes up to 2 min per tube (depending on the leukocyte count of the samples involved). The analysis will take up to 3 min per sample. The entire procedure may be completed within 1 hr for a single sample and within 1.5 hr for a series of ten samples processed simultaneously.

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Wilfried H.B.M. Levering
Henk Wind
Vivian Granger
Kees Sintnicolaas
Herbert Hooijkaas
John T. Reilly
Jan W. Gratama
David Barnett

LONG-TERM STABILIZED BLOOD SAMPLES AS CONTROLS FOR FLOW CYTOMETRIC HLA-B27 SCREENING: A FEASIBILITY STUDY

Submitted

ABSTRACT

Background

Long-term stabilized blood samples are potentially useful as positive or negative procedure controls for flow cytometric HLA-B27 screening, and could serve as test samples in an external quality assessment (EQA) scheme. We evaluated long-term stabilized whole blood specimens as prepared for the UK NEQAS for Leucocyte Immunophenotyping EQA scheme (Sheffield, UK).

Methods

Peripheral blood samples were obtained from 9 blood bank donors with known HLA-B typing. Short-term stabilization with Trans-FIX™ was performed before shipment to Sheffield. Thereafter, long-term stabilization was performed. Commercially available HLA-B27 mAb were tested periodically between 1 week and 12 months on fresh, short-term stabilized, and long-term stabilized blood samples using a stain, lyse & wash technique. We compared the forward scatter (FSC), sideward scatter (SSC), and fluorescence signals of lymphocytes as a function of time. Furthermore, a pilot send-out with stabilized blood samples of 4 blood bank donors was distributed among the participants to the Benelux EQA scheme for HLA-B27 screening and results were compared with historical EQA data obtained using non-stabilized blood samples from the same donors.

Results

There were no major effects on FSC and SSC characteristics of lymphocytes. Background fluorescence of stabilized samples increased as compared to fresh samples, but the discrimination between HLA-B27 positive and HLA-B27 negative samples remained feasible post stabilization. In the pilot send-out the results obtained with stabilized samples were less concordant due to variable quality of these samples.

Conclusions

Long-term stabilized whole blood samples can be useful as true HLA-B27 positive and true HLA-B27 negative control cells for daily and longitudinal quality control of flow cytometric HLA-B27 screening. In the same way, long-term stabilized samples can be used for EQA purposes. However, long-term stabilized samples are only of limited value for reagent validation purposes. Extensive quality control of stabilized samples is necessary before distribution to the laboratories.

INTRODUCTION

Since 1973, a strong association between HLA-B27 and ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system, is known (1,2,3). Nowadays it is evident that HLA-B27 is common to the entire group of seronegative spondyloarthropathies which also include Reiter's disease, subgroups of intestinal and psoriatic arthropathies, subgroups of juvenile rheumatoid arthritis and acute anterior uveitis (4). Also the heart has been identified as target for HLA-B27-associated disease; certain atrioventricular conduction blocks and isolated aortic regurgitation are strongly associated with HLA-B27 (5). Therefore, screening for HLA-B27 is commonly performed as a clinical diagnostic procedure.

Various methods exist for HLA-B27 screening, such as complement dependent cytotoxicity (CDC) assay (6), flow cytometry (FCM) (7), molecular typing based on DNA amplification like the polymerase chain reaction (PCR) with sequence specific primers (PCR-SSP) (8,9), PCR with sequence specific oligonucleotides (PCR-SSO) (10,11), and enzyme-linked immunosorbent assays (12). Alternative methods such as typing using cytotoxic T-lymphocytes as cellular reagents (13), isoelectric focusing (14), serological assessment of HLA-B27 positivity using soluble HLA-antigens in serum (15), allele-specific PCR melting assay (16), and leukocyte agglutination (17) are not routinely used. Flow cytometric HLA-B27 screening is most widely used but DNA-based typing is winning ground. Flow cytometric HLA-B27 screening is hampered by the fact that HLA-B27 belongs to the large HLA-B7 cross-reacting group (CREG) of serologically defined antigens. Within the HLA-B7 CREG, HLA-B13, B22, B27, B40, B41, B42, B47 and B48 share common epitopes with HLA-B7 (18,19). In addition, cross-reactivities of HLA-B27 typing reagents, including murine monoclonal antibodies (mAb), with non HLA-B7 CREG antigens such as HLA-B12, B16, B17, B37 and B73, have been reported (20,21,22,23,24,25,26). As crossreactivity of serological reagents is well known, the European Federation for Immunogenetics (EFI) and the American Society for Histocompatibility (ASHI) have formulated specific guidelines for flow cytometric HLA-B27 screening, with which accredited histocompatibility laboratories must comply (27,28). These guidelines provide detailed procedures for instrument standardization and calibration, and HLA-typing by flow cytometry; for the latter, extensive validation of reagents is required. According to the EFI guidelines cellular controls should be run for each batch of mAb. Such control cells should include at least five cells known to express the specified antigen, two cells for each cross-reacting antigen, and at least two cells lacking the specific and cross-reacting antigens. In addition, each batch of tests must include a cell sample known to express the antigen under study as a positive control. Therefore, control cells are indispensable for verifying the specificity of mAb, for control samples in each run of tests, and for assignment of positive reac-

tions. However, access to appropriate control cells is problematic for most diagnostic laboratories. Therefore, we set out to investigate the suitability of long-term stabilized whole blood preparations as developed by UK NEQAS for Leucocyte Immunophenotyping (Sheffield, UK) for its EQA schemes, for flow cytometric HLA-B27 screening. These samples are known to retain light scatter and immunological staining characteristics of lymphocytes similar to those in fresh whole blood for up to 300 days of storage (29,30,31). As a result, UK NEQAS for Leucocyte Immunophenotyping now uses long-term stabilized whole blood preparations for all their EQA schemes (www.ukneqasli.org). Importantly, the use of long-term stabilized blood samples would obviate the need for express delivery, allow the reissue of test specimens in case of loss, and prove useful for control and validation purposes.

MATERIALS AND METHODS

Blood bank donors

From the HLA-typed donor registry of the Sanquin Blood Bank South West Region (Rotterdam, the Netherlands), 9 donors were selected on the basis of their HLA-B antigens. HLA-B27 positive donors, as well as those expressing known cross-reacting and non cross-reacting HLA-B antigens (21) were included (Table 1). All donors were typed for HLA-A and B using the standard CDC test (6), and by polymerase chain reaction with sequence specific primers (PCR-SSP) (Olerup-SSP™ , Saltsjöbaden, Sweden). Each donor was requested, after informed consent, to donate 18 ml of blood.

Table 1: HLA-B antigens and alleles of selected blood bank donors

ID#	HLA-B phenotyping		HLA-B genotyping		Reactivity HLA-B27 mAb[1]
1	B27	--[2]	B*2705	--	Positive
2	B8	B27	B*08	B*2705	Positive
3	B8	B35	B*08	B*35	Negative
4	B8	B35	B*08	B*35	Negative
5	B7	B57	B*07	B*57	Cross-reactive
6	B37	--	B*37	--	Cross-reactive
7	B7	--	B*07	--	Cross-reactive
8	B38[3]	B39[3]	B*38	B*39	Cross-reactive
9	B55[4]	--	B55	--	Cross-reactive

Table 1: [1] Reactivity of HLA-B27 mAb with HLA-B antigens (See reference [21]); [2] '--' blank (i.e., no other HLA-B allel detected. Donor probably homozygous); [3] HLA-B38 and HLA-B39 are split antigens of the broad antigen HLA-B16; [4] HLA-B55 is a split antigen of the broad antigen HLA-B22.

Stabilization

For shipping purposes, short-term stabilization was performed within 5 hours after collection of freshly drawn Na-heparin anticoagulated whole blood. Short-term stabilization was performed by adding 180 µl TransFix™ (Cytomark Ltd, Buckingham, UK) to 18 ml peripheral blood. The samples were divided in 9 mL aliquots and shipped by overnight courier to UK NEQAS for Leucocyte Immunophenotyping (Sheffield, UK). The samples were long-term stabilized immediately upon their receipt (29,30,31). Thereafter, the samples were sent to Erasmus MC (Rotterdam, the Netherlands) where flow cytometric HLA-B27 screening was performed.

Reagents

We studied the reactivity patterns of the fresh, short-term, and long-term stabilized samples with the following anti-HLA-B27 mAb:

(i) Immunotech (Marseille, F) and Beckman-Coulter (Miami, FL): IOTest HLA-B27 FITC (clone ABC-m3) + HLA-B7 PE, catalog # PN IM1502, and isotypic control IgG2a FITC-IgG1 PE dual color reagent, catalog # PN IM1255.

(ii) BD Biosciences (San Jose, CA): HLA-B27 Kit™, catalog # 340183, containing anti-HLA-B27 mAb clone GS145.2 FITC mixed with the CD3 PE mAb clone SK7.

(iii) One Lambda, Inc. (Canoga Park, CA): HLA-B27 FITC (clone FD705), catalog # B27F50X, and mouse IgG2b FITC conjugated isotype control, catalogue # G2BF50.

Each sample was tested at each occasion against the full set of reagents.

Flow cytometry

Assays were performed on 9 occasions (i.e., after venipuncture, within 5 hours after short-term stabilization, and after long-term stabilization at 1, 2, and 4 weeks, and after 2, 3, 6, and 12 months counted from the moment of venipuncture). The FACSCalibur™ instrument was set up using Calibrite™ beads and FACSComp™ software (all from BD Biosciences). In addition, the appropriate positioning of the FL1 window of analysis in sample space was verified using reference beads (RCP-60; Spherotech, Libertyville, IL). On all occasions the FL1 channel (in which data of all HLA-B27 mAb were collected) was calibrated using Quantum™ FITC low level beads (cat. # 824; Bangs Laboratories, Fishers, IN). Levey-Jennings plots were prepared to monitor the FL1 primary performance parameters (32). These parameters for instrument performance were within the limits of acceptability (33). In this way a standardized FL1 window of analysis is obtained which allows meaningful comparison of median FL1 fluorescence intensity (MFI) results in this longitudinal study. In addition, a separate instrument setup according to manufacturer's instructions was done for the HLA-B27 Kit™ in order to analyze the samples stained with this reagent.

Each reagent was tested as per the manufacturer's instructions. For all HLA-B27 mAb except for clone GS145.2, 50 µl of undiluted blood was added to the recommended amount of mAb and incubated for 20 min at room temperature (RT) in the dark. Then 2 ml of NH4Cl solution was added, followed by vortexing and incubation for 10 min at RT in the dark to lyse the erythrocytes. Following centrifugation for 5 min at 400 x g, the remaining leukocytes were washed twice in 2 ml PBS supplemented with 1% bovine serum albumin (BSA). After removing the supernatant, the cell pellet was resuspended in 0.25 ml FACSFlow™ solution (BD Biosciences). For the HLA-B27 Kit™ , 50 µl of undiluted blood was added to 30 µl GS145.2 FITC/CD3 PE mixture, followed by vortexing and incubation for 15 min at RT in the dark. Then 2 ml of 1x FACS Lysing Solution™ was added followed by vortexing and incubation for 10 min at RT in the dark to lyse the erythrocytes. Following centrifugation for 5 min at 400 x g the remaining leukocytes were washed in 2 ml PBS. After removing the supernatant, the cell pellet was resuspended in 0.25 ml FACSFlow™ solution. Data acquisition and interpretation was performed as previously described (21).

Data management

The following data were collected for each mAb after gating on lymphocytes: (i) the MFI of the isotype control histogram; (ii) the MFI of the anti HLA-B27 mAb histogram; and (iii) the qualitative interpretation of the result (i.e., negative or positive). For the HLA-B27 Kit™, the conclusion obtained with the dedicated software was used. The conclusions on the data of individual mAb were 'negative', 'equivocal', or 'positive'. For the HLA-B27 Kit™, conclusions were 'negative', 'positive' or – in case of problems with autogating - 'no result'.

Proof of principle

To evaluate the performance of long-term stabilized peripheral blood samples within the setting of an EQA for HLA-B27 screening, we selected 4 donors who had donated blood samples for a previous send-out in the setting of the Benelux EQA scheme for HLA-B27 screening (i.e., spring 2003 [sample D, 27 participants], spring 2004 [sample A, 28 participants], and spring 2005 [samples B and C, 30 participants]) (Table 2). These results had been obtained with fresh, non-stabilized samples. To obtain proof of principle, an extra HLA-B27 EQA was organized in 2006 using long-term stabilized peripheral blood samples from the same donors. We compared the results obtained with long-term stabilized blood samples with the historical data. Results are illustrated with data from 1 participant (Figure 5).

Table 2: Proof of principle. EQA results derived from samples, fresh and stabilized

Sample	Outcome	FCM HLA-B27 screening	
(number of participants		Fresh	Stabilized
HLA-B*2705, B*35	Negative	1	3
(A; n=28)	Positive	24 (86%)	14 (50%)
	FTR[2]	3	8
	No result	0	3
HLA-B*07, --[1]	Negative	29 (97%)	26 (87%)
(B; n=30)	Positive	0	0
	FTR[2]	1	2
	No result	0	2
HLA-B*08, B*35	Negative	29 (97%)	27 (90%)
(C; n=30)	Positive	0	0
	FTR[2]	1	1
	No result	0	2
HLA-B*07, B*44	Negative	25 (93%)	14 (52%)
(D; n=27)	Positive	0	1
	FTR[2]	2	4
	No result	0	8

Table 2: [1] '--' blank (i.e., no other HLA-B antigen detected. Donor probably homozygous) [2] Further typing required.

Table 3: Effects of short and long-term stabilization on the assignment of HLA-B27.

ID#	HLA-B genotyping		Interpretation per HLA-B27 mAb[4]								
			ABC-m3 Stabilization			FD705 Stabilization			GS145.2 Stabilization		
			None	Short	Long	None	Short	Long	None	Short	Long
1	B*2705	--[1]	P	P	P	P	P	P	P	P	P
2	B*08	B*2705	P	P	P	P	P	P	P	P	P
3	B*08	B*35	N	N	N	N	N	N	N	N	N
4	B*08	B*35	N	N	N	N	N	N	N	N	N
5	B*07	B*57	P	P	P	N	N	N	N	N	N
6	B*37	--	P	P	P	N	N	N	N	N	N
7	B*07	--	P	P	P	N	N	N	N	N	N
8	B*38[2]	B*39[2]	P	P	E	N	N	N	N	N	N
9	B*55[3]	--	P	P	P	N	N	N	N	N	N

Table 3: [1] '--' blank (i.e., no other HLA-B allel detected. Donor probably homozygous); [2] HLA-B38 and HLA-B39 are split antigens of the broad antigen HLA-B16; [3] HLA-B55 is a split antigen of the broad antigen HLA-B22; [4] Abbreviations : 'P' = positive, 'N' = negative, and 'E' = equivocal.

Note: Long-term stabilization, data collected 1 week after venipuncture.

RESULTS

Matrix effects of short-term and long-term stabilization

The effects of short-term and long-term stabilization on the FSC and SSC signals of lymphocytes (FSC^{low}, SSC^{low}), monocytes ($FSC^{intermediate}$, $SSC^{intermediate}$) and granulocytes ($FSC^{low\ to\ intermediate}$, SSC^{high}) are illustrated using list mode data from sample 2 (Figure 1). The FSC and SSC signals of freshly drawn peripheral blood (*upper left panel*) served as reference. Upon short-term stabilization a slight decrease of FSC and SSC signals of all 3 subsets was observed (*upper right panel*). This reduction was more evident after long-term stabilization at 1 week post venipuncture (*lower left panel*). After gating on FSC and SSC, the autofluorescence of the lymphocytes, as visualized using the FL2 signal, was increased upon long-term stabilization (*lower right panel*).

In the same way, the effect of short-term and long-term stabilization on the FL1 signals obtained with the 3 different HLA-B27 mAb was studied (Figure 2). The 'net FL1 signals' (i.e., FL1 MFI, obtained after subtraction of the isotype control signal (ABC-m3 and FD705) or that of the internal calibrator (GS145.2) from the corresponding experimental results) are shown in Figure 2. After short-term stabilization, slight – if any – increments of 'net FL1 signals' were seen, whilst after long-term stabilization (i.e., after 1 week of storage) more substantial reductions of the 'net FL1 signals' were observed. For the GS145.2 mAb the internal calibrator served as threshold to discriminate between positive and negative results. "Corrected" results for the GS145.2 mAb were <0 if the FL1 signals obtained with this mAb were lower than those of the internal calibrator.

To investigate whether or not these changes in FSC, SSC and 'net FL1 signals' would have an impact on the assignment of HLA-B27 positivity, independent qualitative interpretations were performed by two experienced technicians (see Materials and Methods). These interpretations were fully consistent (data not shown). The 9 selected blood bank donors were either HLA-B27 positive (samples 1 and 2), HLA-B27 negative (samples 3 and 4), or HLA-B27 negative, B7 CREG (i.e., sample 5 to 9; Table 1). Upon long-term stabilization and 1 week of storage, the HLA-B27 negative samples were scored "negative" using the ABC-m3 mAb, and the HLA-B27 positive samples were scored "positive" (Table 3). Under these conditions, 4 of the 5 HLA-B27 negative, HLA-B7 CREG samples (5, 6, 7 and 9) were (falsely) scored "positive", and 1 sample (8) was scored "equivocal". The ABC-m3 mAb is well known for its cross-reactivity with non HLA-B27 antigens (21). The FD705 and GS145.2 mAb yielded fully consistent results between fresh, short-term and long-term stabilized samples (Table 3).

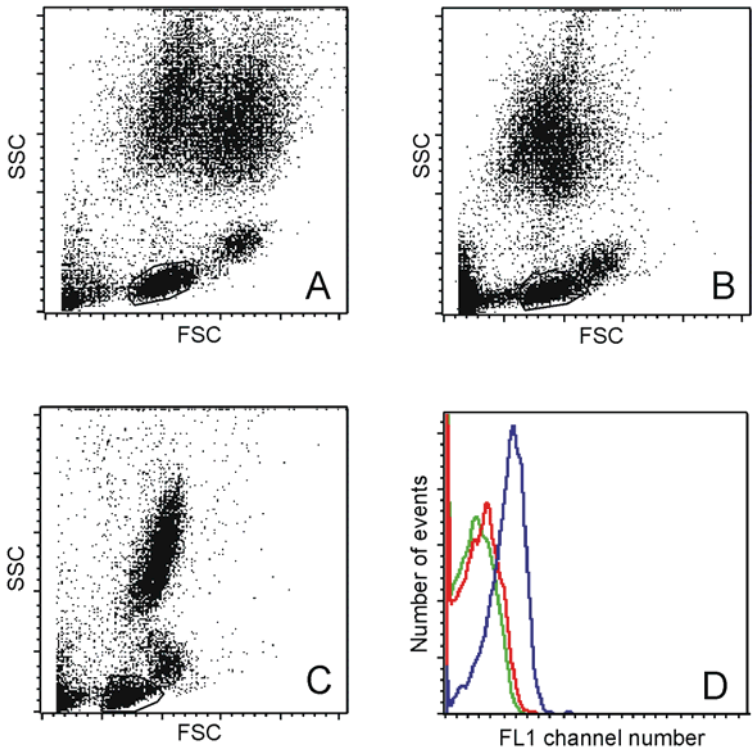

Figure 1: Matrix effects of stabilization. Matrix effects of stabilization on the FSC and SSC characteristics of sample #2 (HLA-B8,27). Freshly drawn peripheral blood (*upper left panel*), short-term stabilized peripheral blood (*upper right panel*), and long-term stabilized (i.e., 1 week after venipuncture) peripheral blood (*lower left panel*). The autofluorescence of lymphocytes is visualised using the FL2 histogram (lower panel right). Green = fresh, red = short-term stabilization, and blue = long-term stabilization.

Stability of long-term stabilized samples

The effects of long-term stabilization as a function of time (i.e., after 1 week to 12 months of storage) on FL1 signals are shown in Figure 3.

Results are shown for all 3 HLA-B27 mAb tested. Data of an HLA-B27 negative sample (i.e., sample 3), an HLA-B27 negative, HLA-B7 CREG sample (i.e., sample 5), and an HLA-B27 positive sample (i.e., sample 2) are shown. The FL1 histograms of freshly drawn peripheral blood (*upper row*) served as benchmark. Short-term stabilized peripheral blood (*second row*) showed minor increments of the FL1 signals of the isotype control mAb (i.e., IgG2a FITC, and IgG2b FITC) as compared to that of unstained cells (autofluorescence). A gradual increment of the FL1 signals of isotype control mAb continued upon storage up to 12 months (*third to ninth rows*). In con-

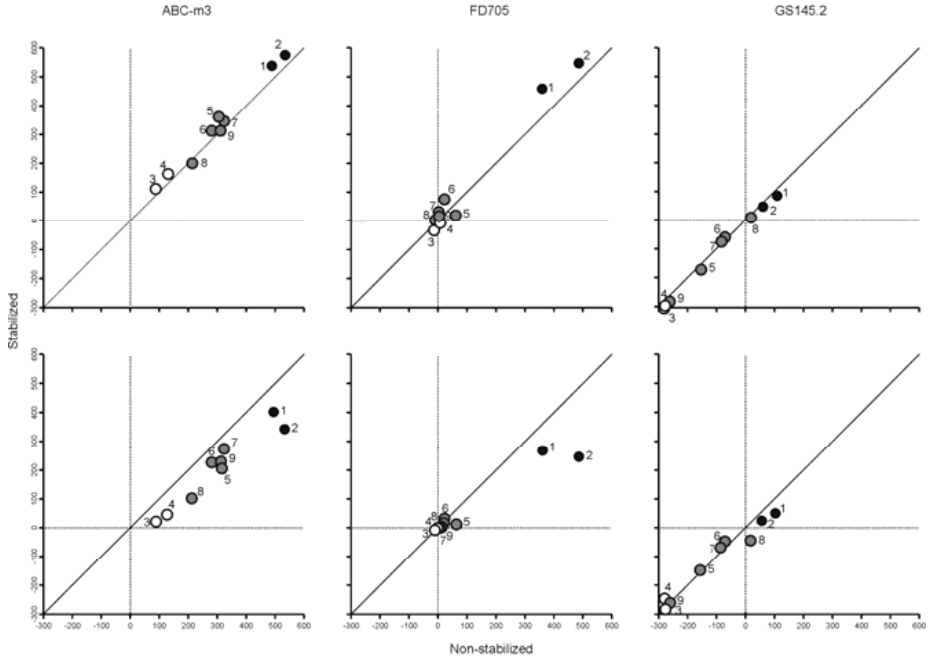

Figure 2: Effects of stabilization on the 'net FL1 signal'. Effects of stabilization on the 'net FL1 signal' of the anti-HLA-B27 mAb ABC-m3 (*left column*), HLA-B27 mAb FD705 (*middle column*), and HLA-B27 mAb GS145.2 (*right column*). Results shown are the 'net FL1 signals' (i.e., FL1 MFI, obtained by subtracting the results of the corresponding isotype controls (ABC-m3 and FD705) or internal calibrator (GS145.2) from the experimental results). Results of freshly drawn samples are plotted along the horizontal axes, and those of short-term (*upper panels*) and long-term stabilization (i.e., 1 week after venipuncture) (*lower panels*) along the vertical axes. The line y=x is shown as a reference. Each symbol represents a cell donor and the labels of the symbols correspond with their unique identifiers (ID#) in Table 1. HLA-B27 positive samples are depicted with black symbols, HLA-B27 negative samples with open symbols, and HLA-B27 negative, B7 CREG samples with grey symbols.

trast, the FL1 histograms of anti-HLA-B27 mAb (i.e., ABC-m3, FD705 and GS145.2) showed slight reductions in intensity upon stabilization and storage. This pattern was already visible after short-term stabilization (second row), and became more evident upon storage of long-term stabilized samples up to 12 months (third to ninth rows). The 'net FL1 signals' are shown in Figure 4. During long-term stabilization and storage up to 12 months, some reduction of 'net FL1 signals' was observed, but the results obtained with the 3 anti-HLA-B27 mAb on HLA-B27 negative, HLA-B27 negative, B7 CREG and HLA-B27 positive samples remain similar.

The impact of long-term stabilization and storage during 1 week, 1 month, 3 months, 6 months and 12 months on the assignment of HLA-B27 positivity is shown in Table 4.

Table 4: Effects of long-term stabilization and storage on the assignment of HLA-B27.

ID#	Interpretation per HLA-B27 mAb[1]														
	ABC-m3 Stabilization					FD705 Stabilization					GS145.2 Stabilization				
	1wk	1mo	3mo	6mo	1yr	1wk	1mo	3mo	6mo	1yr	1wk	1mo	3mo	6mo	1yr
1	P	P	P	P	P	P	P	P	P	P	P	NR	NR	NR	NR
2	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
4	N	N	N	N	N	N	N	N	N	N	N	N	NR	N	N
5	P	P	P	P	E	N	N	N	N	N	N	N	N	N	N
6	P	P	P	P	P	N	N	N	N	N	N	N	N	N	N
7	P	P	P	P	P	N	N	N	N	N	N	N	N	NR	NR
8	E	E	E	N	N	N	N	N	N	N	N	N	NR	NR	NR
9	P	P	P	E	E	N	N	N	N	N	N	N	N	N	N

Table 4: [1]Abbreviations : 'P' = positive, 'N' = negative, 'E' = equivocal, and 'NR' = no result.

Using the anti-HLA-B27 mAb ABC-m3, the HLA-B27 negative samples (3 and 4) were scored "negative" after long-term stabilization and up to 12 months of storage. Similarly, the HLA-B27 positive samples (1 and 2) were scored "positive" during that time. Two HLA-B27 negative, B7 CREG samples (6 and 7) were consistently scored (falsely) "positive" after long-term stabilization. The remaining 3 HLA-B27 negative, B7 CREG samples (5, 8, and 9) yielded inconsistent classifications upon long-term storage. Sample 5 was scored "positive" up to 6 months and "equivocal" at 12 months; sample 8 was scored "equivocal" at 1 week and 1 and 3 months, and "negative" at 6 and 12 months; and sample 9 was scored "positive" at 1 week and 1 and 3 months, but "equivocal" at 6 and 12 months. These changes in classification as a function of storage time reflected the loss of specific fluorescence signal obtained with the anti-HLA B27 mAb upon storage (Figure 4). In contrast, the results obtained with the FD705 mAb were consistent with those obtained on fresh samples (Table 3) and were not affected by storage time of the samples (Table 4).

Similar to the FD705 mAb, results with the GS145.2 mAb were consistent with those obtained on fresh samples (Table 3) and were not affected by storage time up to 12 months (Table 4). However, the results became more variable when the list mode data were analyzed with the software provided with the HLA-B27 Kit™. This situation was due to failure of the 'autogating' procedure based on the FSC and FL2 (CD3-PE) char-

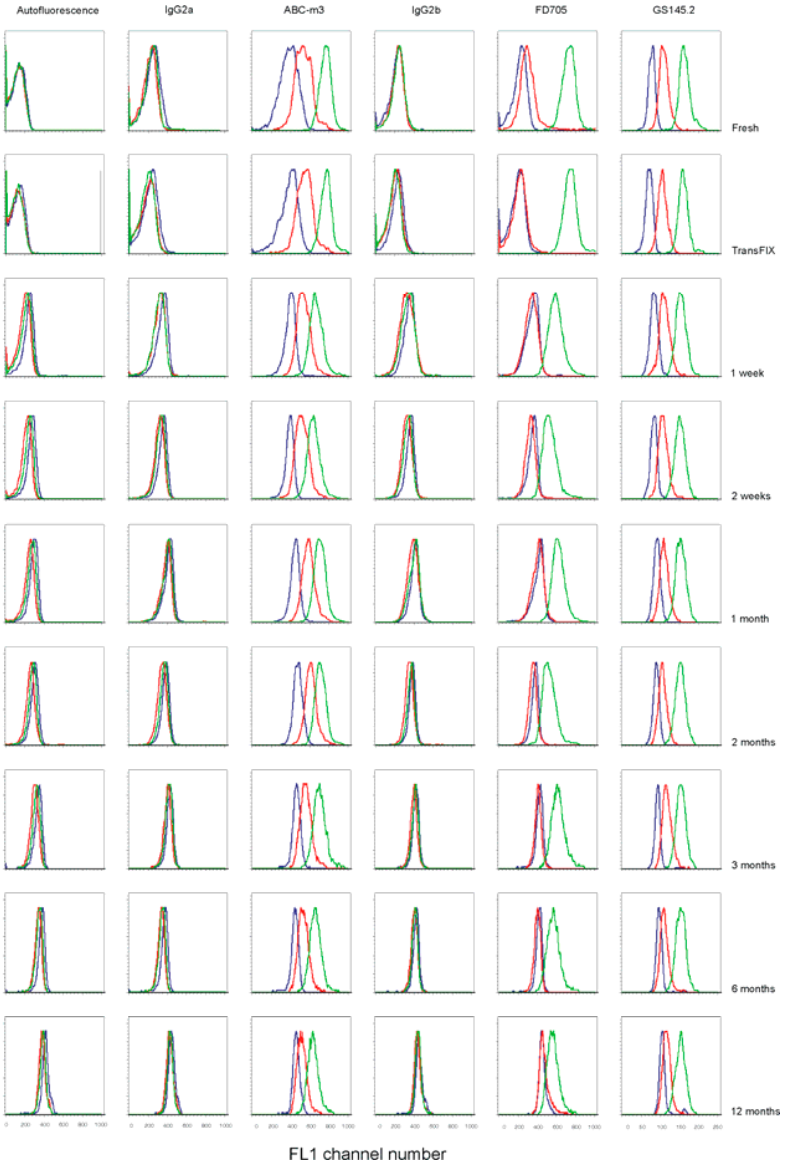

Figure 3: Results obtained from a HLA-B27 negative, HLA-B27 positive, and HLA-B cross-reacting sample. FL1 histograms obtained with the anti HLA-B27 mAb ABC-m3 (*second and third column*), FD705 (*fourth and fifth column*), GS145.2 (*sixth column*), and their corresponding isotype controls. The autofluorescence signal is shown in a FL2 histogram (*first column*). Assays were performed on 9 occasions (i.e., after venipuncture [*first row*], after short-term stabilization [*second row*], and after long-term stabilization at 1, 2, and 4 weeks, and, 2, 3, 6, and 12 months [*third to ninth row*]) (see Materials en Methods). Results of sample #3 (HLA-B8,35; blue line), sample #5 (HLA-B7,57; red line), and sample #2 (HLA-B8,27; green line) are shown.

acteristics of lymphocytes. Autogating failure did not occur after 1 week of storage and only once after 1 month, but was encountered in one third of samples stored between 3 and 12 months. Autogating failed irrespective of HLA-B27 typing of the samples (i.e., positive, negative or cross-reactive). Manual gating on FSC-FL2 (CD3-PE) characteristics of lymphocytes resolved this problem on all occasions (data not shown).

Proof of principle

To explore the feasibility of long-term stabilized samples for use in an HLA-B27 EQA, four blood bank donors were selected (Table 2). After 47 days of storage, long-term stabilized samples of these donors were distributed in the setting of the regular EQA program for HLA-B27 screening to 41 participants (20), and are referred to as Donors A, B, C and D. Sample collection and stabilization was performed as described in Materials and Methods. The FSC and SSC characteristics and the FL1 histograms obtained with the 3 HLA-B27 mAb on fresh and long-term stabilized samples of Donor D are shown in Figure 5. Again, the 'net FL1 signal' decreased slightly upon long-term stabilization. Table 2 compares the results of assignment of HLA-B27 positivity on fresh and long-term stabilized samples by the participants.

Whilst 2 of the 28 to 30 participants reported poor sample quality for the stabilized samples from Donors A, B and C, 9 of 27 did so for Donor D. Figure 5 (*lower panels*) shows the deteriorated FSC and SSC characteristics of Sample D as compared to Sample A. Several participants reported poor red cell lysis of Sample D, which is illustrated as a cluster of events with very low FSC and SSC signals (Figure 5). In addition, Sample D, but not Sample A, showed – for unknown reasons - bimodal FL1 fluorescence histograms which were most outspoken for the FD705 mAb.

A comparison of results on long-term stabilized vs. fresh samples (Table 2) showed that the best concordance between both sample types was obtained for Donor B (HLA-B27 negative, B7 CREG; 87% vs. 97% correct negative results) and Donor C (HLA-B27 negative; 90% vs. 97% correct negative results). Donor A (HLA-B27 positive) stood out by a relatively low proportions of correct positive results on fresh (86%) and long-term stabilized (50%) samples. Failure to obtain a positive result was due to lack of results (n=3), inconclusive results (n=8) or incorrect negative results (n=3). Finally, excellent results had been obtained on fresh samples from Donor D (HLA-B27 negative, B7 CREG; 93% correct negative results), whilst only 52% of results were correct on stabilized samples from this donor. Failure to obtain correct results on Donor D's stabilized blood were due to lack of results (n=8), inconclusive results (n=4) or incorrect positive result (n=1). Taking the results on stabilized samples of all 4 donors together, the levels of concordance between fresh and long-term stabilized samples did not appear to correlate with HLA-B27 type, but rather with the quality of the long-term

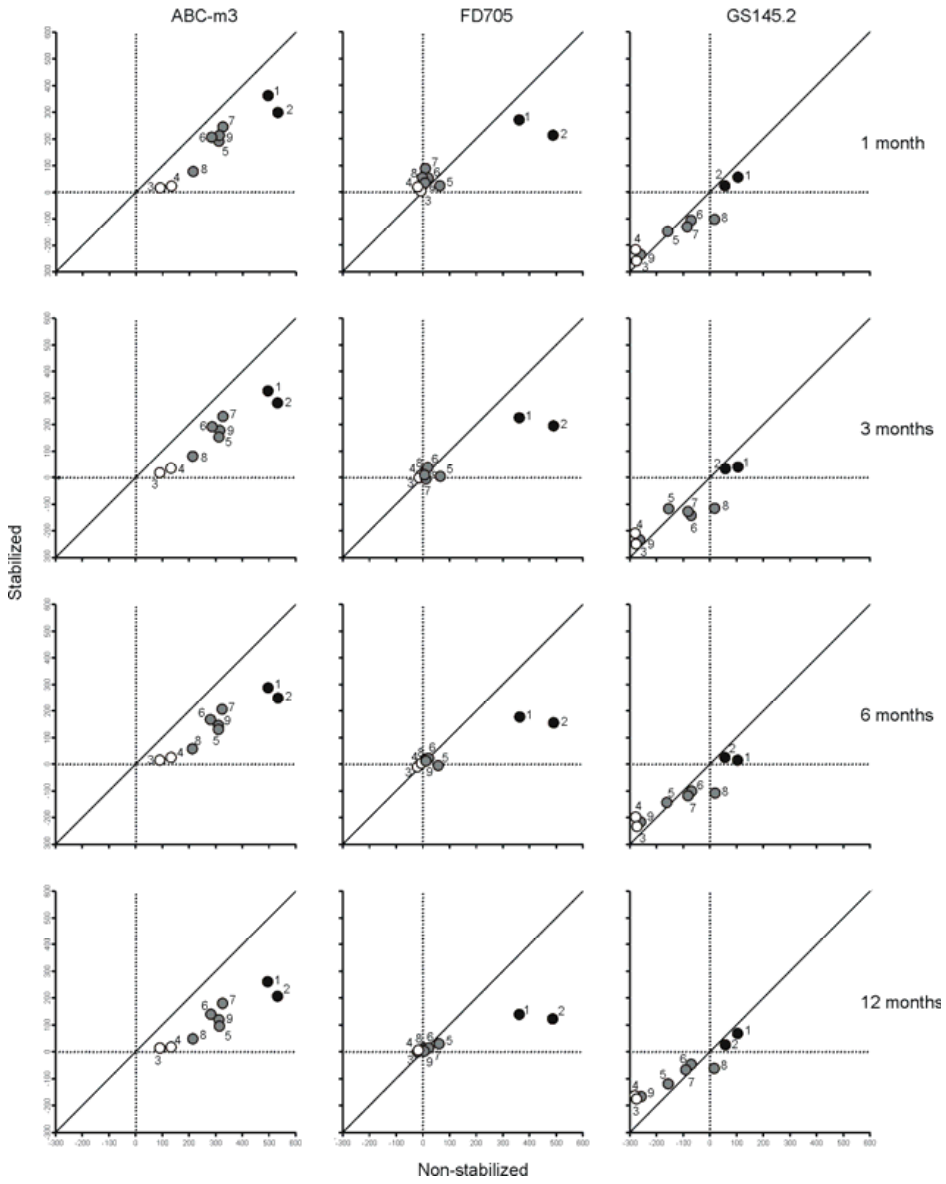

Figure 4: Effects of long-term stabilization on the 'net FL1 signal'. Effects of long-term stabilization on HLA-B27 assignment using anti-HLA-B27 mAb ABC-m3 (*left column*), HLA-B27 mAb FD705 (middle column), and HLA-B27 mAb GS145.2 (*right column*). The y- and x-axis reflect the FL1 MFI, corrected for the corresponding isotype control (ABC-m3 and FD705) or beads (GS145.2). Results shown are the 'net FL1 signals'. Results of 1 month (*first row*), 3 months (*second row*), 6 months (*third row*), and 12 months (*fourth row*) long-term stabilization are plotted against the corresponding results of freshly drawn samples. See further the legend to Figure 2.

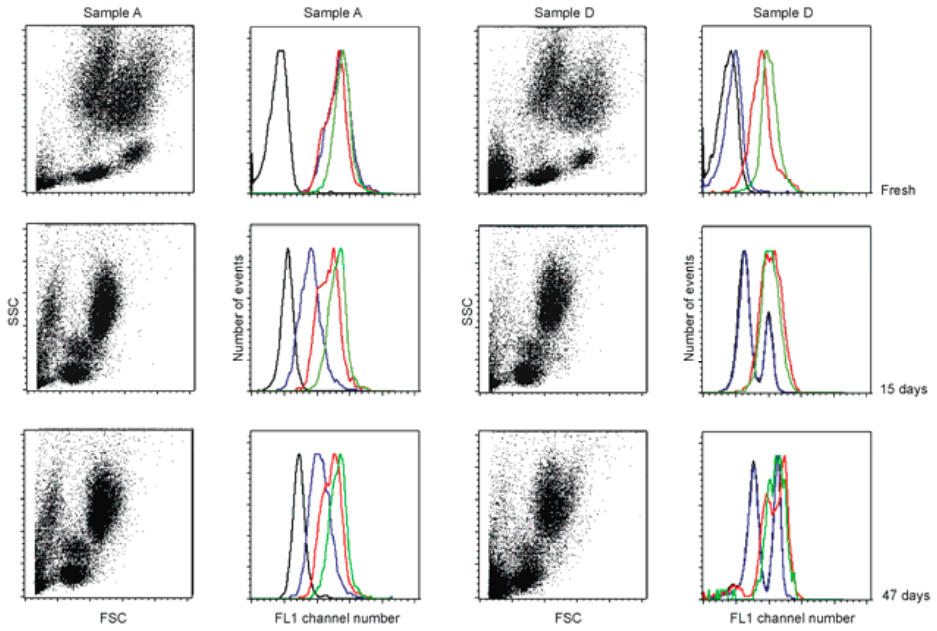

Figure 5: Representative results obtained from flow cytometric HLA-B27 screening EQA. FSC-SSC dotplots and FL1 histograms obtained with sample A (first and second column) and sample D (third and fouth column) as recorded by the coordinating laboratory. Assays were performed on 3 occasions (i.e., on the day of venipuncture [first row], shortly after long-term stabilization (i.e., 15 days after venipuncture) [second row], and after send-out (i.e., 47 days after venipuncture) [third row]) (see Materials en Methods). The FL1 histograms show: IgG1 isotype control (black line), anti-HLA-B27 mAb ABC-m3 (red line), FD705 (blue line), and GS145.2 (green line).

stabilized specimens. Finally, 5 out of 9 participants performing DNA-typing were not able to isolate DNA for PCR-SSP techniques. All 5 used QIAamp™ DNA Blood Kits (Qiagen, Hilden, Germany). The remaining 4 participants used MagNA Pure™ Nucleic Acid Isolation Kits (n=2), High Pure™ PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland; n=1), and Chemagic™ DNA Blood Kit (Chemagen AG, Baesweiler, Germany; n=1). As complement-depending cytotoxicity requires the use of viable leukocytes, both participants using this technique were unable to report results on stabilized samples.

DISCUSSION

Flow cytometric HLA-B27 screening EQA is typically performed on fresh blood samples. The limitations of fresh whole blood as test material for EQA purposes are as follows: fresh, nonstabilized samples require rapid distribution, preferably within 24 h (20,34). There is no guarantee that such samples will arrive in a similarly good condition as they were dispatched. The limited stability of fresh whole blood precludes shipment of repeat specimens of the original sample in case of irregularities. Over time, untreated cells tend to undergo changes in light scatter characteristics as a result of apoptosis. For example, discrete white blood cell populations in untreated whole blood could no longer be resolved based on FSC and SSC properties after 7 days of storage (35). The recovery of CD3+, CD4+, and CD8+ T cells in untreated whole blood had declined from 100% to 30% after 10 days of storage (36). In line with these observations, appropriate assessment of absolute CD3+, CD4+, and CD8+ T-cell counts is not possible after 3 to 4 days of storage of untreated peripheral blood at room temperature (37,38). There are no data available on the stability of HLA-B27 and B7 CREG expression by lymphocytes in untreated samples over time. However, long-term stabilization of whole blood, as performed by UK NEQAS for Leucocyte Immunophenotyping, has been shown to reduce sample deterioration in EQA exercises for lymphocyte subset enumeration and CD34+ cell counting (31,35,36,39,40,41,42,43).

Current guidelines for flow cytometric HLA-B27 screening (27,28) require extensive validation of reagents and test procedure (i.e., procedure controls for each batch of mAb, and test control cells for each batch of tests). Although most clinical laboratories routinely performing flow cytometric HLA-B27 screening do not comply with these guidelines, an increasing proportion among them do follow the internationally imposed ISO directives (e.g., ISO 17025, ISO 15189). Similarly, the ISO guidelines require general procedure validation and procedure controls for tests performed on ISO-accredited laboratories. For reagent validation and assay control purposes, freshly obtained blood samples with known HLA-B27 typing results are optimal, which, however, are very difficult to obtain for most routine diagnostic laboratories. Cryopreserved and thawed mononuclear cell suspensions may be used, provided that the thawed cells show reactivity patterns with the HLA-B27 screening reagents similar to those of the same cells when tested freshly. No major difficulties in HLA-B27 assignment have been reported when flow cytometric HLA-B27 typing was performed on fresh and cryopreserved and thawed mononuclear cells (24). Currently, a few manufacturers (e.g., Axxora Life Sciences (San Diego, CA) and Phoenix Flow Systems (San Diego, CA)) provide HLA-B27 positive control cells that are stabilized preparations of human cell lines. Although these are of some value as positive control cells in flow cytometric HLA-B27 screening, neither cryopreserved and thawed MNC nor cell lines are true

procedure controls for whole blood and therefore do not provide full process control for flow cytometric HLA-B27 screening.

In this study, we have analyzed the light scatter characteristics, autofluorescence, and cell surface density of HLA-B27 antigens of short-term and long-term stabilized whole blood samples. We addressed the feasibility of the long-term stabilized samples [i] as control cells (i.e., HLA-B27 positive and negative control cells for each batch of assays), [ii] for the validation of reagents used in the procedure of flow cytometric HLA-B27 screening, and [iii] as test samples for EQA of flow cytometric HLA-B27 screening. In general, the light scatter characteristics of lymphocytes remained similar to those observed with fresh whole blood, allowing their identification based on FSC and SSC characteristics for up to 12 months. The autofluorescence signal increased immediately upon short-term stabilization, and continued to do so during 12 months of storage. In contrast, the HLA-B27 expression (read out in FL1) of HLA-B27 positive cells decreased after long-term stabilization resulting in lower 'net FL1 signals' compared to those in fresh samples.

Feasibility of long-term stabilized blood samples as source of test control cells.

Despite the reduction of 'net FL1 signals' upon long-term stabilization, the HLA-B27 assignment of cells classified as positive or negative by reference HLA typing (i.e., serological reactivity) was similar to those in fresh samples up to 12 months. The signal threshold for each anti-HLA-B27 mAb to assign positive reactions must be established for each individual laboratory. In this study, the mean fluorescence intensity (MFI) of the long-term stabilized HLA-B27 positive samples remained higher then those of long-term stabilized cells with negative or cross-reacting serological reactivities. Therefore, the assignment of the minimum reactivity of a positive reaction is feasible. The MFI of cells with negative serological reactivities remained lower or equal to those of cells with cross-reacting reactivities. From this point of view, the use of long-term stabilized samples with positive and negative serological reactivities is feasible as positive and negative test control cells for each batch of tests with a shelf life up to 12 months.

Feasibility of long-term stabilized blood samples for reagent validation.

Cells expressing HLA-B antigens belonging to serological cross-reacting groups are essential to verify the specificity of mAb. The HLA-B27 assignment of long-term stabilized cells with cross-reacting serological reactivities was similar to those of fresh cells for up to 3 months. In addition, the FL1 MFI of the long-term stabilized samples was, in general, lower than those of fresh cells. Therefore, our current experience is that

long-term stabilized blood samples do not meet the strict requirements for reagent validation purposes, and fresh samples should continued to be used for this purpose.

Feasibility of long-term stabilized samples for EQA of flow cytometric HLA-B27 screening.

In our pilot study, 2 samples showed similar results for fresh and stabilized samples, 1 sample was problematic with low 'net FL1 signals', and 1 sample was suboptimal because of deteriorated FSC-SSC characteristics. Review of the results of our entire study revealed that the latter problem rarely occurred as lymphocytes of the other 12 samples in our study showed normal FSC-SSC characteristics. Thus, long-term stabilized samples are feasible for EQA purposes with the same caveats as for the test control cells and procedure controls. Extensive quality control of stabilized samples is necessary before distribution to the laboratories to avoid problems with poor FSC-SSC characteristics and decrease of the 'net FL1 signals' obtained with HLA-B27 flow cytometric typing reagents

In conclusion, we demonstrated the suitability of a long-term stable whole blood preparation for use as true positive and true negative (i.e., non cross-reacting) HLA-B27 control cells for both daily and longitudinal quality control of flow cytometric HLA-B27 screening. In the same way, long-term stabilized samples can be used for EQA purposes. However, based on our current experience, long-term stabilized samples are only of limited value for reagent validation purposes (i.e., to analyze cells expressing cross-reacting HLA-B antigens). Given these limitations, the procedure for long-term stabilization of blood samples developed by UK NEQAS for Leucocyte Immunophenotyping is a promising first step to the production of control cells for flow cytometric HLA-B27 screening.

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Wilfried H.B.M. Levering
Frank W.M.B. Preijers
Wessel N. van Wieringen
Jaco Kraan
Wil A.M. van Beers
Kees Sintnicolaas
Dick J. van Rhenen
Jan W. Gratama

FLOW CYTOMETRIC CD34+ STEM CELL ENUMERATION: LESSONS FROM NINE YEARS' EXTERNAL QUALITY ASSESSMENT WITHIN THE BENELUX COUNTRIES

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ABSTRACT

Background

A biannual external quality assurance (EQA) scheme for flow cytometric CD34+ haematopoietic stem cell enumeration has been operational in the Benelux countries since 1996. In an evaluation of the results of 16 send-outs, we studied the effects of the methods used on assay outcome and whether or not this exercise was effective in reducing between-laboratory variation.

Methods

Data were analyzed using robust multivariate regression. This approach is relatively insensitive to outliers and is used to assess the effect of methodological aspects of CD34+ cell counting on the bias and variability.

Results

Five variables were associated with significant bias of absolute CD34+ cell counts: (i) unique laboratory number (ULN), (ii) gating strategy; (iii) CD34 mAb fluorochrome; (iv) type of flow cytometer, and (v) method of sample preparation. In addition, ULN and platform methodology (i.e., single vs. dual) contributed significantly to the variability of this assay. Overall, the variability in results of CD34+ cell enumeration has declined with time; in particular, after a practical workshop in which participants were trained to use the "single platform ISHAGE protocol".

Conclusions

Between-laboratory variation in CD34+ cell enumeration can be reduced by standardization of methodologies between centres. Our approach, i.e., EQA with targeted training and feedback in response to reported results, has been successful in reducing the variability of CD34+ cell enumeration between participants

INTRODUCTION

Haematopoietic stem cell (HSC) transplantation has been successfully used to reconstitute hematopoiesis after myeloablative therapies in man since the late 1950s (1,2). HSCs have the capability of homing into the marrow microenvironment and regenerating multi-lineage haematopoiesis in relatively short time (3). Nowadays, many clinical studies have established that transplantation of CD34+ stem cells is a potentially curative therapy for many patients with malignant and non-malignant haematological diseases (4–6). Initially, autologous and allogeneic HSC transplants have been performed utilizing bone marrow HSCs (7). In the last decade, increasing numbers of transplantations have been carried out using HSCs from mobilized peripheral blood (PB) or umbilical cord blood. Haematopoietic recovery is dependent on the number as well as the colony-forming capacity of the transplanted HSCs. This emphasizes the need for an adequate prediction of engraftment potency of HSCs in patients after receiving myeloablative therapy followed by HSC transplantation (8–11). Furthermore, enumeration of CD34+ cells has been shown to be useful in considerations of peripheral blood stem cell harvesting (12–16) and in comparing the efficacy of various PB stem cell mobilization strategies (reviewed by Fruehauf and Kessinger and Sharp) (17,18). Furthermore, CD34+ cell enumeration is useful for monitoring the stem cell yield during graft manipulations, such as immunomagnetic selections, ex vivo expansion, or genetic modifications of HSCs. The advent and use of a CD34 monoclonal antibody enabled the detection of these cells by flow cytometry (19). Several flow cytometric studies have shown a direct correlation between the CD34+ cell content of PB in patients mobilized with chemotherapy (with or without growth factors such as granulocyte colony stimulating factor [G-CSF]) and the number of CD34+ cells collected by apheresis. Additionally, the number of transplanted HPCs as defined by flow cytometry was shown to correlate better with engraftment post SCT than that assessed using colony-forming unit culture assays (20). Initial enumeration methods for CD34+ cells (19–24) have been improved by distinguishing viable from nonviable cells (25,26). To ensure that CD34+ cell counts are widely valid for clinical decision making, these must be accurate and precise. Therefore, various national and international guidelines for CD34+ cell enumeration have been formulated (24,26–28). Nevertheless, in various multicentre studies (29–40) large variations between results of individual centers were observed. The outcome of CD34+ cell enumeration assays depends on several variables, such as sample source, sample processing, technical experience, and data analysis techniques. In the Benelux countries, an external quality assessment (EQA) scheme for CD34+ cell enumeration has been in place since 1996. Since then, participants have been trained and standard protocols have been provided (37). In this article we review our experience with this EQA scheme during its first 9

years. We addressed the impact of sample (e.g., origin, preparation, stabilization), flow cytometric CD34+ cell enumeration techniques (e.g., instrumentation, reagents, and analytical strategies), and laboratory experience on assay outcomes. In particular, we were interested in the efficacy of our EQA scheme to improve results, i.e., to reduce the variability and/or bias in CD34+ cell counts between laboratories.

MATERIALS AND METHODS

Study Design

This study consisted of 16 send-outs comprising 64 samples (PB, apheresis products, and cord blood), which were distributed to laboratories that had participated in the biannual EQA scheme for flow cytometric CD34+ cell enumeration organized within the Benelux. This scheme was run under the auspices of the Foundation for Immunophenotyping in Hemato-Oncology (SIHON), the Foundation for Quality Control in Medical Laboratories (SKML; all in The Netherlands), and the Belgian Association for Analytical Cytometry (BVAC/ABCA). The samples were obtained from various sources as shown in Table 1.

Table 1: Overview of 64 Distributed Samples by Origin and Stabilization Method

Sample origin	Shelf life	Stabilization method	*n*
Peripheral blood	n.a.	No stabilization	18
	Short-term	TransFix™	3
		StabilCyte™	3
		WAK chemie	1
		Streck	1
	Long-term	UK NEQAS	17
Apheresis product	n.a.	No stabilization	13
	Short-term	StabilCyte™	5
Cord blood	Short-term	WAK Chemie	3

Table 1: n.a. = not applicable

Short-term stabilization was performed with TransFix™ (Cytomark Ltd, Buckingham, UK) or StabilCyte™ (BioErgonomics, St Paul, MN), and performed by WAK Chemie (Bad Soden, Germany) and Streck (Omaha, NE), respectively. Long-term stabilization was performed by UK NEQAS for Leucocyte Immunophenotyping (Sheffield, UK). The samples were divided in 1-mL aliquots and shipped by overnight courier to the participants. Each participant was requested to: (i) perform flow cytometric CD34+ cell enumeration according local protocols and (ii) provide relevant methodological details.

On some occasions (i.e., 16 samples in 4 send-outs) the CD34+ cell enumeration was performed in triplicate to obtain intra-institutional reproducibility results. Reports of results and methodologies were to be carried out within 14 days upon receipt of the samples. Data analysis for debriefing of the EQA results was performed by the SKML data center. For each sendout, an overall debriefing report was issued and discussed at biannual participant meetings. In the Spring of 2000, the participants were invited to a workshop in which a new standard protocol (ISHAGE single platform) was introduced (37). Participants who were performing insufficiently were offered a hands-on training at the coordinating laboratory (Erasmus MC-Daniel den Hoed).

Data Processing and Parameter Classification

For this study, the results of all CD34+ cell enumerations as well as the responses to the questionnaires were taken into account. Where necessary, incorrect data entries were corrected after consultation with the submitting participants. In addition, printed output of data analyses of the two 2004 send-outs were centrally reviewed to check for inconsistencies in the reported usage of gating strategies. Data processing and statistical analyses were performed using the STATA™ software (StataCorp., College Station, TX) as indicated in the text. Each laboratory was assigned a unique number for referral purposes. The absolute CD34+ cell number was assigned as response variable. The following 13 categorical variables were assumed to influence the outcome of CD34+ cell enumeration assays. These are summarized in Table 2 and discussed in detail later.

EQA send-out.

Each send-out, from Spring 1996 to Autumn 2004 was chronologically assigned with a unique number (i.e., 1–16). In this way, we analyzed any effect of the EQA program on the results of the participants as a function of time.

Laboratory expertise.

As actual figures of the number of performed CD34 enumerations per laboratory per year were not available, a surrogate parameter for laboratory expertise was used. We classified the laboratories into three mutually exclusive groups based on estimated experience: (i) laboratories that had only participated in the our EQA scheme but had had no daily practice in CD34 enumeration in the setting of a SCT program; (ii) laboratories participating in the Dutch Working Party of Stem Cell Laboratories (WSN; an organization of laboratories that perform the processing of stem cell transplants); and (iii) laboratories that were members of the European Group for Blood and Marrow Transplantation (EBMT), but were not members of the WSN. We reasoned that WSN participants were expected to have outstanding experience in CD34 enumeration be-

Table 2: Overview of 13 Categorical Variables

Variable	Categories
EQA send-out	1 – 16[a]
Laboratory expertise	WSN membership
	EBMT membership
	No membership
Workshop 2000 participation	Yes
	No
Sample source	Peripheral blood
	Apheresis product
	Cord blood
Sample stabilization	No stabilization
	Short term stabilization
	Long term stabilization
Gating strategy	Milan
	Bender
	ISHAGE
	SIHON
	ProCOUNT™ (BD Biosciences)
	Stem-Kit™ (Beckman-Coulter)
Platform methodology	Single
	Dual
Flow cytometer	FACScan™
	FACScalibur™
	FACStar™
	Epics XL™
	Cytoron™
Haematology analyser	Technicon-Bayer
	Sysmex
	Beckman-Coulter
	Abbott
	Other
Beads	TruCOUNT™ (BD Biosciences)
	FlowCount™ (Beckman-Coulter)
	Perfect Count™ (Cytognos)
	Volumetry
	Unknown
CD34 monoclonal antibody (mAb)	HPCA-1 [My10] (BD Biosciences)
	HPCA-2 [8G12] (BD Biosciences)
	581 (Coulter-Immunotech,
	IQ products, and Sanquin Diagnostics)
	Birma-K3 (DAKO)
Labeling CD34 mAb	FITC
	PE
	APC
	None
Sample preparation	Lyse and Wash
	Lyse No Wash
	No Lyse No Wash
	Mononuclear Cells

Table 2: [a] Send-outs were numbered sequentially as a function of time.

cause of the regular exchange of information on this issue between these laboratories, whilst EBMT members had only had an active stem cell transplantation program.

Participation at "Workshop 2000".

This workshop, featuring a hands-on training in CD34+ cell enumeration, was organized for all participants in the Benelux CD34 EQA scheme. In this workshop, the single-platform ISHAGE method for CD34+ cell enumeration was introduced and recommended as the state-of-the-art technique (37). We analyzed the effects of this educational activity on systematic differences and variability in CD34+ cell enumeration.

Sample source.

Three different sample sources were distinguished: (i) PB; (ii) apheresis product; and (iii) cord blood. We expected that the sample source would affect the quality and complications of CD34+ cell enumeration. For example, apheresis products may contain relatively high proportions of platelets and dead or dying (CD34+) cells; cord blood suspensions usually contain high proportions of nucleated red blood cells (RBC).

Sample stabilization.

We expected that if the samples were stabilized before shipment, the variation between laboratories in preventing sample decay would be reduced. We distinguished three categories: (i) no stabilization, (ii) short-term stabilization, and (iii) long-term stabilization.

Gating strategies.

We distinguished six strategies (methods); (i) Milan; (ii) Bender; (iii) ISHAGE; (iv) SIHON; (v) ProCOUNT™; and (vi) Stem-KIT™.

The *Milan protocol* is a "whole blood, stain-lyse-wash" method (20,22). The gating procedure is based on selection of leukocytes as denominator set on forward scatter (FSC) and side scatter (SSC), excluding debris and aggregates. Within these leukocytes, a positive fluorescence analysis region is set on cells with $SSC^{low\ to\ intermediate}$ in a control sample stained with an isotype-matched control mAb. Within this fluorescence analysis region, the number of events of the CD34 mAb-stained samples are counted and used as numerator in the calculation of %CD34+ cells.

The *Bender protocol* (21) is the first multicolor analytical strategy in which CD45 fluorescein isothiocyanate (FITC) is included as a leukocyte marker in addition to CD34 phycoerythrin (PE) to eliminate nonleukocytes and debris from the analysis and to generate a stable denominator. CD45 staining is used to establish a more stable and precise denominator by including only nucleated white blood cells in the analysis.

CD45+ events are then analyzed in a similar manner to the Milan protocol using an isotype control and CD34 staining versus SSC analysis to enumerate CD34+ cells.

The *ISHAGE protocol* (24) utilizes the maximum information available of four parameters; forward-light and side-light scatter and the intensity of CD34 and CD45 staining. These four parameters were combined in a sequential Boolean gating strategy that can be used to enumerate HSCs from a variety of sources.

An alternative sequential Boolean gating approach is the *SIHON protocol* of the Dutch Foundation for Immunophenotyping in Hemato-Oncology (SIHON) (36). Here, the laser dye solution LDS-751 that stains DNA and to a lesser degree RNA, is used to identify nucleated cells and to exclude debris, platelets, and unlysed erythrocytes. The denominator is nucleated cells (i.e., LDS-751^{bright}). To exclude the effects of Fc□ receptor mediated mAb binding by monocytes and granulocytes from subsequent analyses, PE-conjugated antibodies to CD14 and CD66e are used to gate out these cell types. Thereafter, a region is set on the CD34+, SSC^{low} cluster, identified using a class III antibody labeled with FITC, which also is used for the analysis of the isotype control. Any events stained by the isotype are subtracted from the CD34+ test result.

The *ProCOUNT*™ single-platform kit by BDBiosciences (San Jose, CA) is based on counting beads (TruCOUNT™ tubes). The denominator for nucleated cells is a nucleic acid dye (NAD). A threshold is set on nucleated cells and during software-driven data acquisition, sufficient events are recorded to ensure a 10% precision in absolute CD34+ cell counts. Data analysis is also software-driven. The gating strategy is aimed at identifying CD34+ cells; first by gating on NAD^{pos}, SSC$^{low\ to\ intermediate}$ events and, second, by gating on CD45$^{neg\ to\ normal\ positive}$ events. Within these events the CD34^{pos} events are analyzed. A similar analysis is run for the isotype-matched control. The TruCOUNT™ counting beads are gated on their bright fluorescent signals they emitted in the FL1, FL2, and FL3 channels.

Finally, *Stem-KIT*™ from Beckman Coulter (Miami, FL) consists of a two-color fluorescent CD45 FITC + CD34 PE, and CD45 FITC + isotonic control PE murine mAb reagent, the nucleic acid viability dye 7-amino-actinomycin-D (7-AAD), NH_4Cl lysing reagent, and Stem-Count™ fluorospheres (Beckman-Coulter) to directly generate absolute CD34+ cell counts. Analysis was performed in a similar way to the single-platform ISHAGE protocol (25). Automated enumeration is also possible with the Stem-ONE™ software (Beckman Coulter).

Platform methodology.

(i) Single and (ii) dual platform methods were distinguished. The use of single platform methodologies are expected to reduce the between-laboratory variation in CD34+ cell enumeration by eliminating the leukocyte differential count from additional haematology analyzer as a source of variation.

Flow cytometric instrumentation.
As there were major differences between the various instruments in use, we investigated this parameter as a possible source of variation.

CD34 mAb clone.
We distinguished four categories: (i) My10 (HPCA-1), (ii) 8G12 (HPCA-2), (iii) 581, and (iv) Birma-K3. All these mAb except My10 (Class I) were of Class III (41).

CD34 mAb fluorochrome.
We assigned two categories: (i) FITC and (ii) PE. We expected that the fluorochrome brightness (i.e., PE > FITC) would affect between-laboratory variation of CD34+ cell enumeration.

Sample preparation.
Four categories were distinguished: (i) Lyse and wash (LW); (ii) Lyse no wash (LNW); (iii) No lyse no wash (NLNW); and iv) gradient separation of mononuclear cells (MNC). Variable cell losses, due to different sample preparation techniques, would affect between-laboratory variation.

Statistical Analysis

First, a descriptive analysis of raw data of CD34+ cell enumerations was performed. This analysis revealed that the measurements had a low variability at low cell counts and a high variability at high cell counts, which is often observed with cell enumeration data. As standard statistical techniques require an approximately constant variability over the whole cell count range, the data were logarithmically transformed.

To assess the effect of multiple variables on the logtransformed CD34+ cell enumeration data, robust multivariate regression was used (42). This approach is less sensitive to outliers than standard multivariate regression analysis. We then addressed two aspects of the quality of the log-transformed CD34 measurements: bias (i.e. systematic differences) and variability (i.e. random differences) in separate analyses. Analysis of the mean of the log-transformed data revealed which variables caused systematic differences (bias) in the mean CD34+ cell counts. Subsequently, the bias was removed; the residuals of this analysis were used to investigate the variability of the CD34+ cell counts. To this end, the absolute values of these residuals (termed absolute error, which is

related to the standard deviation) was used. A robust multivariate regression analysis of the absolute error of the log-transformed data was then performed to assess which variables affected the variability of CD34+ cell counts. For both multivariate analyses a step-down procedure was followed, i.e., at each iteration of the analysis the least

significant variable was removed. This procedure was continued until all nonsignificant variables were removed. Similar analyses were performed on percentages CD34+ cells to check for inconsistencies in procedures and outcomes (data not shown).

RESULTS

Methods Used and Change of Usage Patterns with Time

From 1996 to 2004, 56 laboratories participated to 16 send-outs in our EQA scheme (between 37 and 44 participants per send-out). The central review of printed output regarding the reported gating strategies of the two 2004 send-outs showed no major inconsistencies. One participant used an alternative Boolean gating strategy for the ISHAGE approach, one participant used the ISHAGE approach instead of the reported Bender approach, one participant used the Bender approach instead of the reported Milan approach, and one participant used an additional CD45 mAb in the SIHON approach. Regarding the ISHAGE approach, 95% (19/20) of the participants applied the sequential Boolean gating strategy as described in Ref. 24. In general, we could assume that the reported information regarding the gating strategies is consistent during the survey period. Of the 64 distributed test samples, 43 were blood specimens that had either not been stabilized (n = 18), short-term stabilized (n = 8), or long-term stabilized (n = 17). Long-term stabilized samples have mostly been distributed since the Spring of 2002. Eighteen test samples had been derived from apheresis products (13 non-stabilized, 5 short-term stabilized) and the remaining three test samples were short-term stabilized cord blood specimens (Table 1). For analysis of list-mode data, various gating strategies have been used (Fig. 1, panel A). The use of less sophisticated analytical strategies tends to decline with time: (i) the Milan approach (from 21% in 1996 to 5% of participants in 2004), (ii) the Bender approach (11% in 1996 and 11% in 2004), and (iii) the SIHON approach (from 55% in 1996 to 11% in 2004).

In contrast, methods based on sequential gating strategies became more widely used with time: (i) the ISHAGE approach (from 13% in 1996 to 55% in 2004), (ii) the Stem-KIT™ assay (from 0% in 1996 to 11% in 2004), and (iii) the ProCOUNT™ assay (from 0% in 1996 to 7% in 2004). As shown in Figure 1, panel B, the use of FITC as fluorochrome for the CD34 mAb by laboratories decreased from 55% in 1996 to 5% in 2004. Consequently, the use of PE as fluorochrome by laboratories increased from 39% in 1996 to 95% in 2004. To establish absolute counts (Fig. 1, panel C), only 5% of the laboratories had adopted the single platform technique in 1996, which had increased to 53% of the laboratories in 2004. As a result of the workshop held

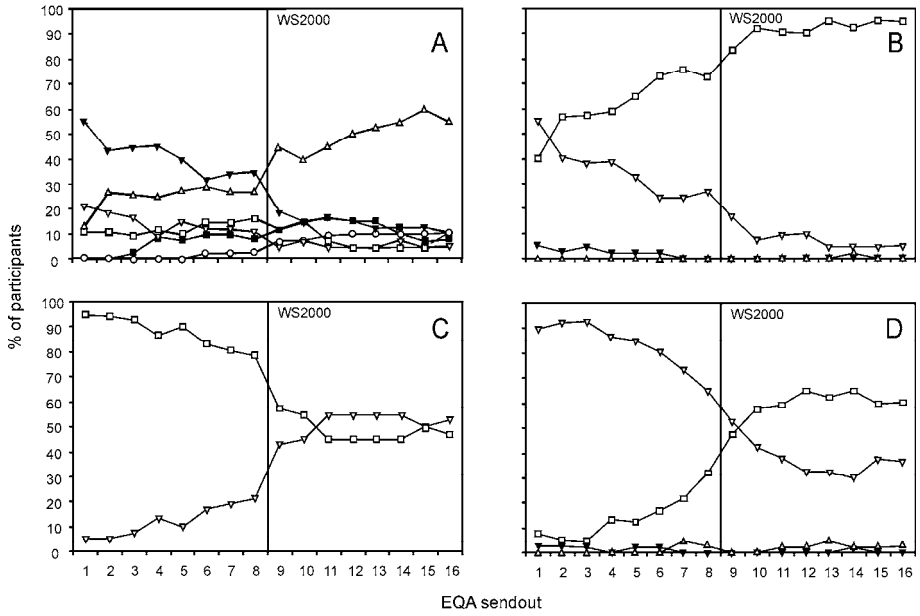

Figure 1: Change of methodology usage patterns over time. Panel A, labeling gating strategies: ▽ = Milan, □ = Bender, △ = ISHAGE, ▼ = SIHON, ■ = ProCOUNT™, O = Stem-KIT™. Panel B, labeling fluorochrome CD34 mAb: ▽ = FITC, □ = PE, △ = APC, ▼ = none. Panel C, labeling platform techniques: ▽ = Single platform, □ = Dual platform. Panel D, labeling sample preparation: ▽ = Lyse and wash, □ = Lyse no wash, △ = No lyse no wash, ▼ = MNC. WS2000: the vertical line indicates the timing of the educational workshop during which the single-platform ISHAGE protocol was introduced to the participants of this EQA scheme (see Materials and Methods).

in the Spring of 2000, in which 75% (42/56) of the participants were introduced to and trained in the ISHAGE single platform technique (37), the number of laboratories using single platform techniques clearly increased. For sample preparation (Fig. 1, panel D), the use of "LNW" methods increased with time at the expense of those of "LW" methods. With time, all single platform users and a small proportion of dual platform users adopted the "LNW" method preparation. As for laboratory expertise, 45% (25/56) participants had no daily practice in CD34 enumeration, 18% (10/56) participants were members of the WSN and participated in EBMT, and 55% (31/56) of participants participated in EBMT only. The participants operated mainly flow cytometers manufactured by BD Biosciences (FACScan™ [from 68% in 1996 to 11% in 2004], FACSCalibur™ [from 5% in 1996 to 53% in 2004], and FACStar™ [from 13% in 1996 to 5% in 2004]), followed by Beckman-Coulter (Epics XL™ [from 8% in 1996 to 34% in 2004]) and Ortho (CytoronAbsolute™ [from 11% in 1996 to 0% in 2004]).

In dual platform techniques, haematology analyzers from Technicon-Bayer (from 21% in 1996 to 5% of participants in 2004), Sysmex (from 21% in 1996 to 16% in 2004), Beckman-Coulter (from 26% in 1996 to 16% in 2004), and Abbott (from 3% in 1996 to 8% in 2004) have been used. In single platform techniques, TruCOUNT™ beads (BD Biosciences [range 3–11% of participants]), FlowCount™ beads (Beckman-Coulter [range 3–18%]), Perfect Count™ (Cytognos [3%]), unknown beads (range 3–20%), and volumetry (range 2–3%) have been used. The majority of the laboratories used clone HPCA-2 (BD Biosciences) as CD34 mAb (range 76–93% of participants), followed by clone 581 (Coulter-Immunotech, (Beckman Coulter, Miami, FL), IQ-products (IQ-products, Groningen, The Netherlands), and Sanquin Diagnostics (Sanquin Diagnostics, Amsterdam, The Netherlands) (range 7–24%).

Table 3: Multivariate Analysis of Factors with Systematic Effects (bias) on Absolute CD34+ Cell Count Results

Variable (overall *P*)	Categories	*P*-value
Gating strategy (<0.01)	Milan	<0.01
	Bender	n.s.
	ISHAGE	n.s.
	SIHON	n.s.
	ProCOUNT™	n.s.
	Stem-Kit™	<0.01
Laboratory (<0.01)	n.a.	
Labeling CD34 mAb (0.01)	FITC	<0.01
	PE	n.s.
Flow cytometer (0.02)	FACScan™	n.s.
	FACScalibur™	0.03
	FACStar™	n.s.
	Epics XL™	n.s.
	Cytoron™	0.04
Sample preparation (0.03)	LW	n.s.
	LNW	0.05
	NLNW	n.s.
	MNC	n.s.

Table 3: Only categorical variables with significant effects are shown.
n.a., not applicable; n.s., not significant.

Factors Affecting the Outcome of CD34+ Cell Enumerations

We studied which of the 13 categorical variables (Table 2) significantly influenced the outcome of CD34+ cell count measurements. The five variables having significant effects are shown in Table 3 and are discussed below.

Gating strategy (Fig. 2, panel A).

Most observations have been made using the ISHAGE gating strategy. As a result, this strategy was used as benchmark and assigned a factor value of 1. In comparison, two strategies stood out by yielding significantly lower outcomes: the Milan method and the Stem-Kit™ assay. The outcomes of the remaining three strategies, i.e., Bender, SIHON, and ProCOUNT™, were not significantly different from that of ISHAGE.

Fluorochrome (Fig. 2, panel B).

PE was most commonly used as fluorochrome. FITC-labeled CD34 mAb yielded significantly lower results than PE-labeled CD34 mAb. A similar pattern was observed when CD34+ cell enumeration results were expressed as proportions of leukocytes, indicating that the effect of fluorochrome was independent of the technique of absolute cell count generation (data not shown).

Flow cytometric instrumentation (Fig. 2, panel C).

Most of the results were obtained using FACScan instruments. In comparison, significantly higher results were obtained using FACScalibur and Cytoron instruments; the latter stood out as a remarkably wide variation. A similar pattern was observed when CD34+ cell enumeration results were expressed as proportions of leukocytes, indicating that the effect of the type of instrument was independent of the technique of absolute cell count generation (data not shown).

Sample preparation (Fig. 2, panel D).

Most of the results were obtained using "LW" methods. In comparison, significantly higher results were obtained using "LNW" methods, whilst the results obtained with "NLNW" methods and those obtained using mononuclear cell isolation were not significantly different.

The effects of the remaining eight categorical variables (Table 2) on the outcomes of CD34+ cell enumerations were not significant.

Laboratory (as defined by ULN; Fig. 3).

Laboratory no. 22 was chosen as benchmark because it had no missing observations. Relative to this laboratory, six other laboratories stood out by systematically higher results (indicated with arrows). A search of possible explanations for these outlying results yielded that laboratory no. 38 had participated only on a few occasions in the EQA program (i.e. 5 send-outs) and hence may have accumulated only limited experience, and that laboratory no. 11 used a nonstandard gating strategy (i.e. SIHON method extended with CD45 gating).

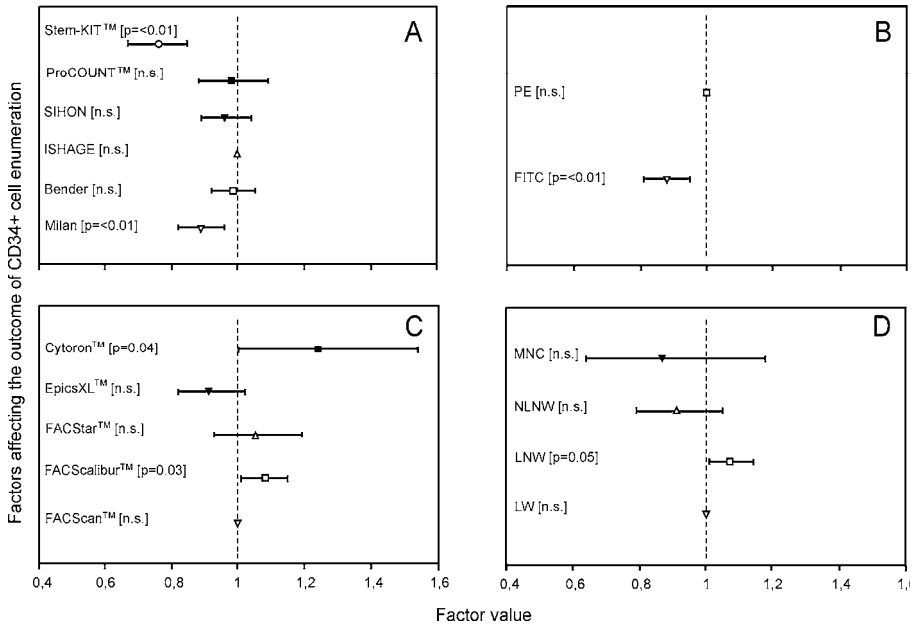

Figure 2: Factors significantly affecting the outcome of CD34+ cell enumeration. Panel A: gating strategies. Panel B: fluorochromes CD34 mAb. Panel C: flow cytometric instrumentation. Panel D: sample preparation. Factor value = 1 for the category with the most observations. The factor value mirrors the relative difference of the other categories related to the category with factor value = 1. The line reflects the 95% confidence interval of the estimation of the factor value. The *P*-value is shown between brackets.

Factors Affecting the Variability of CD34+ Cell Enumerations

Thereafter, we studied which of the 13 categorical variables significantly influenced the variability of CD34+ cell measurements. As shown in Table 4, two variables had significant effects.

Table 4: Multivariate Analysis of Factors Influencing Variability of Absolute CD34+ Cell Count Results

Variable (overall *P*)	Categories	*P*-value
Platform methodology (<0.01)	Single platform	n.s.
	Dual platform	0.01
Laboratory (<0.01)	n.a.	

Table 4: Only categorical variables with significant effects are shown.
n.a., not applicable; n.s., not significant.

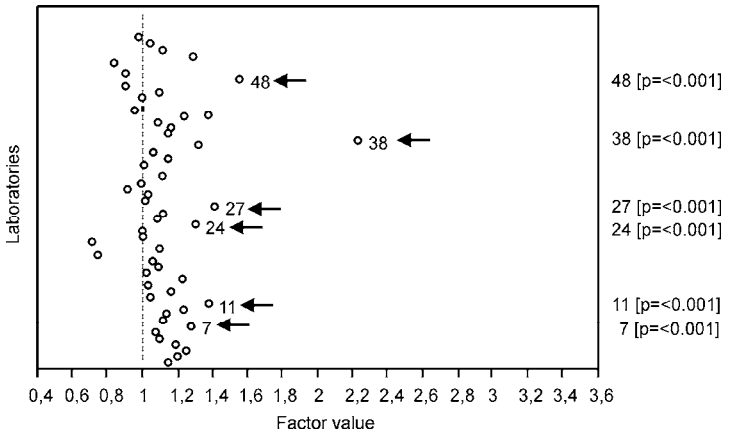

Figure 3: Factors significantly affecting the outcome of CD34+ cell enumeration (continued). Unique laboratory number. Factor value = 1 for laboratory no. 22 which had no missing data ("benchmark"). The factor value mirrors the relative difference of the other categories related to the category with factor value = 1. *P*-values are shown between brackets for laboratories with a significantly devious outcome than the benchmark laboratory.

Platform methodology.

Most of the results were obtained using the single-platform method. Therefore, this category was taken as benchmark. The variability ofCD34+ cell enumerations by dual-platform methods were significantly higher than that of single-platform methods (factor value 1.27 [95% confidence interval 1.05–1.55]).

Laboratory (as defined by ULN; Fig. 4).

Again, Laboratory no. 22 was chosen as benchmark because it had no missing observations. In relation to this laboratory, seven other laboratories stood out by a significantly larger variability in their results (indicated with arrows). One of these labs (no. 38) also stood out by the large systematic difference of its CD34+ cell enumeration results from the benchmark laboratory (Fig. 3). It must be noted that only one of these laboratories was an EBMT member, and none of them had participated in WSN. This information suggests that the seven laboratories with high variability of results were relatively inexperienced (see Materials and Methods).

EQA send-out number (Fig. 5).

The first send-out was chosen as benchmark. The variability of results obtained in send-outs 2–7 did, by and large, not significantly differ from that of the first send-out. From sendout 8 onwards, significantly smaller variations were observed as compared to the first send-out except for send-out 12. The standardization workshop (37), in

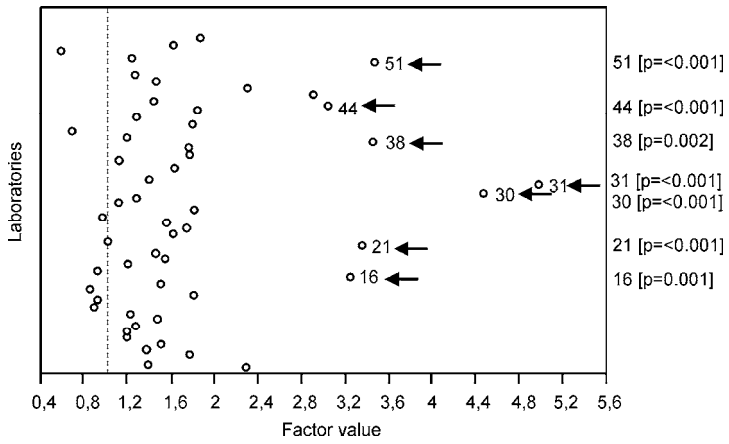

Figure 4: Analysis of the variability of CD34+ cell enumeration. Unique laboratory number. Factor value = 1 for laboratory no. 22 which had no missing data ("benchmark"). The factor value mirrors the relative difference of the other categories related to the category with factor value = 1. *P*-values are shown between brackets for laboratories with significantly higher variability than the benchmark laboratory.

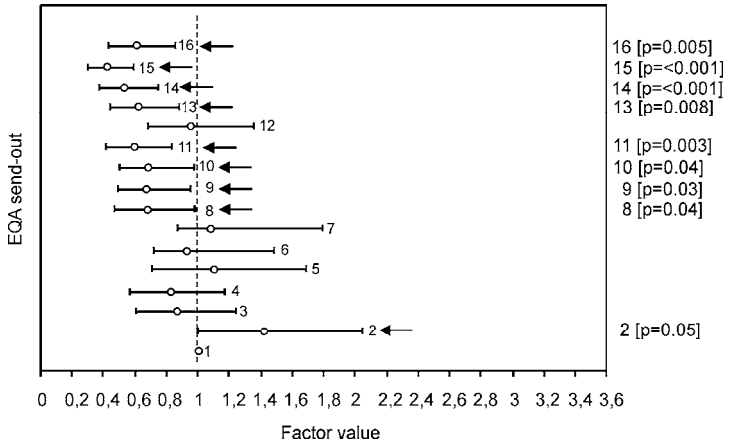

Figure 5: Analysis of the variability of CD34+ cell enumeration by EQA send-out. Factor value = 1 for the first EQA send-out in Spring 1996 ("benchmark"). The factor value mirrors the relative difference of the other send-outs related to the benchmark send-out. The horizontal lines reflect the 95% confidence intervals of the estimation of the factor value. *P*-values are shown between brackets for send-outs with significantly higher variability than the benchmark send-out.

which most laboratories in this EQA scheme had participated, was held between the distribution of send-outs 8 and 9. Seven of the 8 "postworkshop" send-outs had a relatively low variability in CD34+ cell counts (indicated by factor value < 0.8; Fig. 5)

versus only 1 of the 8 "preworkshop" send-outs (Fisher's 2-sided $P = 0.01$). This result suggests that the workshop had been effective in reducing the variability of CD34+ cell enumeration.

DISCUSSION

An EQA program for flow cytometric CD34+ HSC enumeration has been operational in the Benelux countries since 1996. The results of nearly 3,000 assays reported by up to 56 laboratories, in the context of methodological information provided in questionnaires that were issued with each send-out, have been analyzed. We were specifically interested in identifying factors affecting the outcome of CD34+ cell enumeration (i.e., bias) and in identifying factors influencing the variability of this assay. Last but not least, we focused on the efficacy of the EQA program to reduce between-laboratory variation of CD34+ cell enumeration as a function of time.

The influence of 13 variables on the outcome of CD34+ cell enumeration was studied. Besides the individual laboratories, four other variables had significant effects: (i) gating strategy; (ii) labeling of CD34 mAb; (iii) flow cytometer type; and (iv) sample preparation.

Among the factors influencing the variability of CD34+ cell enumeration, platform methodology (i.e., single vs. dual) had significant impact, whilst significant differences in variability between individual laboratories were also observed. Importantly, the variability of CD34+ cell enumeration also declined significantly with time. The effects of the remaining variables, i.e., laboratory expertise, workshop-2000 participation, sample source, sample stabilization, make of haematology analyzer, beads manufacturer, and clone of CD34 mAb, on bias or variability of the absolute CD34+ cell count were not significant.

Among the gating strategies, the Milan and Stem-KIT™ approaches generated significantly lower CD34+ cell counts in comparison to other approaches. With the Milan approach, the selection of leukocytes as denominator is based on FSC and SSC. Light scatter alone cannot exclude debris, RBC, and platelets, which increase the denominator. Furthermore, only bright CD34+ cells were counted, whilst dim CD34+ cells were not included. These findings may explain why lower absolute counts of CD34+ cells were obtained with the Milan gating strategy as compared to other strategies that also included dim CD34+ cells. Another explanation would be the use of an isotype control mAb in the Milan approach. When calculating absolute CD34+ cell counts, events reactive with the isotype control mAb are often subtracted from the experimental result. The effect of this subtraction is relatively large when the numbers of CD34+ cells are low and those of isotype control mAb reactive cells relatively high (34). However, we

had expected a similar effect for the Bender and SIHON approaches in which isotype control mAb-reactive cells were also subtracted, but this was not the case. We do not have an explanation for the relatively low CD34+ cell counts observed with the Stem-KIT™ gating strategy, which is similar to that of the ISHAGE protocol; therefore, similar CD34+ cell counts would have been expected with both approaches. The four participants using the Stem-KIT™ gating strategy also used Stem-KIT™ reagents but not the dedicated analysis software (Stem-ONE™). Inspection of the data analysis files from these laboratories did not reveal methodological inconsistencies. Furthermore, the four participants did not have another methodological feature (such as type of flow cytometer) in common. In this context it should be noted that the use of the ISHAGE method with Flow-Count beads (i.e., similar to the Stem-Kit protocol) yielded similar outcomes as the dual-platform ISHAGE method.

As for labeling of CD34 mAb, the FITC conjugates yielded lower absolute CD34+ cell counts than the PE conjugates. This difference may be caused by the fact that PE emits stronger fluorescent signals and therefore allows better separation between positive and negative populations than does FITC. This notion is confirmed by our observation that the use of PE-conjugated CD34 mAb not only yielded higher absolute counts of CD34+ cells but also larger proportions of CD34+ cells within the leukocytes in comparison to FITC (data not shown).

With the FACScalibur™ and Cytoron™ flow cytometers, relatively high CD34+ cell counts were obtained in comparison to other instruments (i.e., FACScan™, FACStar™, and Epixs XL™). As a volumetric instrument, the CytoronAbsolute™ is less suitable for enumeration of samples with low CD34+ cell counts as it can only acquire and analyze a limited volume of sample and cells in a single run (43). The wide 95% confidence interval for this instrument is due to the low number of observations. We do not have an explanation for the systematically higher CD34+ cell counts observed with FACScalibur™ instruments as compared to FACScan™ instruments, as their optical systems are highly similar. FACScalibur™ instruments are generally newer than FACScan™ instruments, which may have resulted in a somewhat reduced sensitivity for dim CD34+ populations.

The LNW technique generated slightly higher absolute CD34+ cell counts compared to the LW technique; washing may have resulted in (selective) cell losses. The use of NLNW was relatively infrequent, and therefore associated with a wide 95% confidence interval. A problem with this approach may be underestimation of CD34+ cell counts because of the large proportions of red cells in the unlysed samples. The use of MNC separation was discouraged because of the risk of selective cell loss (22), and therefore generally abandoned after the first send-outs. Therefore, only few observations with this method were made resulting in a wide 95% confidence interval.

It is well known that dual-platform techniques are associated with higher variability than single platform techniques (29,37,44). First, single platform technologies bypass the denominator issue, i.e., the percentage CD34+ cells should be reported as a proportion of leukocytes, total nucleated cells, or total events scattering above a FSC threshold. Second, single platform methodologies avoid the variability arising from hematology analysers used to enumerate total nucleated cells or leukocytes (26). Third, single platform methodologies avoid inaccuracies by rounding up or down low percentages of CD34+ cells in dual platform techniques used for calculations of absolute CD34+ cell counts. Our current analysis of 9 years' experience with EQA for CD34+ cell counting confirms and extends the studies by Barnett *et al*, 2000; Chang *et al*, 2004; and Gratama *et al*, 2003. Furthermore, it shows that the implementation of single platform methodology is highly effective in reducing between-laboratory variability of this assay.

An analysis of bias and variability of CD34+ cell counts as a function of individual laboratories (as defined by ULN) revealed that some laboratories stood out by obtaining relatively high CD34+ cell counts, and others by obtaining a relatively large variation in CD34+ cell counting results. As the participants had not been requested to keep flow cytometric list mode data on file for this purpose, it was not possible to retrace the causes of bias and variability for this study.

Finally, we addressed whether or not the CD34 EQA was effective in reducing the variability of CD34+ cell enumerations over a period of time. Indeed, this analysis revealed that the variability in results of CD34+ cell enumeration in this EQA program had declined with time. As most send-outs with a relatively small variability in CD34+ cell counts fell after the 2000 standardization workshop, it seems reasonable to state that this exercise has been effective in reducing between-laboratory variability.

In conclusion, our 9-year EQA exercise of CD34+ cell enumeration has been highly successful in reducing the variability of CD34+ cell enumeration between participants. A major factor has been that a large percentage of participants have adopted a common approach which was—and still is—considered as the state-of-the-art methodology, i.e., the single-platform ISHAGE protocol. Crucial in this respect was the organization of a practical workshop in which this protocol was introduced to the participants, followed by targeted training and feedback in response to reported results. Whilst the goal of the Benelux EQA program for CD34+ cell enumeration has primarily been educational, results of EQA programs will, in general, increasingly serve as a basis for laboratory accreditation. With these regulatory aspects coming into practice, it is imperative that the EQA programs themselves meet the rigorous quality demands. These demands include documentation of the quality of distributed samples and the use of validated procedures for evaluating results. For this purpose, international collaboration should facilitate the development of uniform and statistically appropriate methods which, at

the same time, should allow for comparable results between the different laboratories and the monitoring of a laboratory's poor individual performance, where necessary.

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Wilfried H.B.M. Levering
Wessel N. van Wieringen
Jaco Kraan
Wil A.M. van Beers
Kees Sintnicolaas
Dick J. van Rhenen
Jan W. Gratama

FLOW CYTOMETRIC LYMPHOCYTE SUBSET ENUMERATION: 10 YEARS OF EXTERNAL QUALITY ASSESSMENT WITHIN THE BENELUX COUNTRIES

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ABSTRACT

Background

A biannual external quality assessment (EQA) scheme for flow cytometric lymphocyte immunophenotyping is operational in the Benelux countries since 1996. We studied the effects of the methods used on assay outcome, and whether or not this EQA exercise was effective in reducing between-laboratory variation.

Methods

Eighty test samples were distributed in 20 biannual send-outs. Per send-out, 50 to 71 participants were requested to enumerate CD3+, CD4+ and CD8+ T cell, B cells and NK cells, and to provide methodological details. Participants received written debriefings with personalized recommendations after each send-out. For this report, data was analyzed using robust multivariate regression.

Results

Five variables were associated with significant positive or negative bias of absolute lymphocyte subset counts: (i) platform methodology (i.e., single-platform assays yielded lower CD4+ and CD8+ T-cell counts than dual-platform assays); (ii) sample preparation technique (i.e., assays based on mononuclear cells isolation yielded lower T-cell counts than those based on red cell lysis); (iii) gating strategies based on CD45 and sideward scatter gating of lymphocytes yielded higher CD4+ T-cell counts than those based on "backgating" of lymphocytes guided by CD45 and CD14); (iv) stabilized samples were generally associated with higher lymphocyte subset counts than non-stabilized samples, and (v) laboratories. Platform methodology, sample stabilization, and laboratories also affected assay variability. With time, assay variability tended to decline; this trend was significant for B-cell counts only. In addition, significant bias and variability of results, independent of the variables tested for in this analysis, was also associated with individual laboratories.

Conclusions

In spite of our recommendations, participants tended to standardize their techniques mainly with respect to sample preparation and gating strategies, but less with absolute counting techniques. Failure to fully standardize protocols may have led to only modest reductions in variability of results between laboratories.

INTRODUCTION

Enumeration of the major lymphocyte subsets (i.e., CD3+, CD4+, CD8+ T cells, CD19+ B cells, and CD3-, CD56+ NK cells) yields important information for diagnosis and monitoring of a variety of conditions affecting the immune system. The discovery of human immunodeficiency virus (HIV) in the 1980s as the causative agent of the destruction of CD4+ T cells leading to the acquired immunodeficiency syndrome (AIDS) was the major drive behind the evolution of flow cytometry from research tool to routine diagnostic technique (reviewed in 1). Progressive depletion of CD4+ T cells is associated with an increased likelihood of severe HIV disease and an unfavorable prognosis (2,3). CD4+ T-lymphopenia is also associated with opportunistic infections in recipients of allogeneic hematopoietic stem cell transplants (4) and is also a risk factor for skin cancer in renal transplant recipients (5).

Accurate and reliable measures of CD4+ T cells are important as a quantitative tool for immune status assessment and for health care management of persons infected with HIV (6,7). CD4+ T-cell counts are a criterion for categorizing (i.e., category 1: greater than or equal to 500 cells/μL ; category 2: 200-499 cells/μL ; and category 3: less than 200 cells/μL) HIV-related clinical conditions according to the classification system of the Centers for Disease Control (CDC) for HIV infection and the surveillance case definitions of AIDS among adolescents and adults (8). These categories guide diagnostic and therapeutic actions in the management of HIV-infected adolescents and adults. The need for standardized approaches and quality control procedures in the clinical setting prompted the development of guidelines for CD4+ T-cell enumeration that also cover related topics (e.g., specimen collection, specimen transport, maintenance of specimen integrity, specimen processing, laboratory safety, quality control, quality assessment, and data analysis) (9,10).

In addition to CD4+ T-cell counts, CD8+ T-cell counts are relevant. CD8+ T cells suppress HIV-1 replication. The CD8+ T cells can be activated and increase in counts – inversely to CD4+ T cells – in patients with progressive HIV infection (11). Furthermore, CD8+ T-lymphocytosis is a hallmark of primary immune responses to cytomegalovirus (CMV) and Epstein-Barr virus in otherwise healthy carriers (12), and is associated with recovery from reactivated CMV infection in renal and SCT recipients (13,14). Also, monitoring of CD4+ and CD8+ T-cell counts may provide predictive and prognostic information in patients with metastatic melanoma receiving chemoimmunotherapy (15).

Furthermore, lymphocyte subset enumeration is an important part of the diagnostic workup in patients with acute lymphoblastic and myeloid leukemias (ALL and AML), lymphomas, and chronic lymphoproliferative disorders (16). In addition, serial monitoring of lymphocyte subsets allows the evaluation of treatment efficacy in patients

with post-transplant lymphoproliferative disorders (17). Another application of lymphocyte subset enumeration is to monitor the effectiveness of nutrition supplements in patients receiving peritoneal dialysis (18).

Since the 1990s, several protocols for flow cytometric lymphocyte subset enumeration have been developed (reviewed in 19). In response to the increased dependence of clinical decision-making on this assay, various (inter)national guidelines for lymphocyte subset enumeration, as well as external quality assessment (EQA) schemes, have been set up. Until now, a large variability between results of individual centers has been observed in multi-center studies (20-27). In 1995, we organized a pilot multi-center study in Belgium, The Netherlands, and Luxemburg ('Benelux' countries) as an introduction to a biannual EQA scheme. Here, we review our experience with this scheme during its first 10 years. We addressed the impact of various methodological aspects on assay outcomes with the aim to identify the main sources of between-laboratory variation, and to document the efficacy of our EQA scheme to reduce this variation.

MATERIALS AND METHODS

Study design

We evaluated 20 send-outs comprising 80 peripheral blood samples distributed to laboratories that participated in the biannual EQA scheme for flow cytometric immunophenotyping organized within the Benelux. This scheme was run under the auspices of the Foundation for Immunophenotyping in Hemato-Oncology (SIHON), the Foundation for Quality Control in Medical Laboratories (SKML; both in The Netherlands), and the Belgian Association for Analytical Cytometry (BVAC/ABCA). After informed consent, 73 patients and 3 apparently healthy donors each donated 100 ml of EDTA-anticoagulated venous blood. Seventy-five samples were distributed without stabilization; one sample from a healthy donor underwent short-term stabilization using StabilCyte™ (BioErgonomics, St Paul, MN). Three of the 4 remaining samples were either discarded units of blood for transfusion that had been long-term stabilized by UK NEQAS for Leucocyte Immunophenotyping (Sheffield, UK) (28) or a commercial stabilized blood preparation (Ortho AbsoluteControl™; Ortho-Clinical Diagnostics [Raritan, NJ]). The clinical diagnoses associated with the 73 patient samples were: status after allogeneic hematopoietic stem cell transplantation (n=18); B-chronic lymphocytic leukemia (B-CLL; n=13); severe fatigue (n=11); B-cell non-Hodgkin's lymphoma (n=7); monoclonal B-cell population with undetermined significance (n=4); T-large granular lymphocytic leukemia (T-LGL; n=4); leukocytosis (n=3); multiple myeloma (n=2); and acute Epstein-Barr virus infection, acute myeloid leukemia, anemia, angio-immunoblastic

lymphadenopathy, bladder carcinoma, Burkitt's lymphoma, eosinophilia, polyclonal B-cell lymphocytosis, rheumatoid arthritis, severe aplastic anemia and T-cell non-Hodgkin's lymphoma (n=1 each).

Except for the patients with B-CLL or T-LGL, none had expansions of phenotypically abnormal CD3+, CD4+ or CD8+ T cells, B cells or NK cells. In the latter 2 groups of patients, the abnormal lymphocytes did not preclude the enumeration of CD3+, CD4+, CD8+ T cells, B cells and NK cells using standard protocols. The samples were selected so as to include all possible abnormalities in leukocyte counts and/or proportions of lymphocytes; in only 23 samples (29%), both parameters were normal (Table 1).

Table 1: Overview of 80 distributed samples by leukocyte count and lymphocyte proportion.

Absolute numbers of leukocytes	Proportion of lymphocytes	Number of samples
Low	Low	7
Low	Normal	5
Low	High	3
Normal	Low	11
Normal	Normal	23
Normal	High	11
High	Low	5
High	Normal	3
High	High	12

Table 1: Normal range of absolute number of leukocytes, 4.0 - 10.0 x 109/L, normal range of proportion of lymphocytes (i.e., percentage of leukocytes), 15 - 40%.

The samples were divided in ~1.5 mL aliquots and shipped by overnight courier to the participants. Each participant was requested to perform lymphocyte subset enumeration according to its routine protocol and to answer a questionnaire on methodological details. Data processing and analysis for anonymous debriefing of the EQA results was centrally performed by the SKML datacenter. For each send out, an overall debriefing report was issued to all participants, and discussed at biannual participant meetings. In addition, each participant received an individual report of its data with specific comments and recommendations in case of outlying results. In spring 2001, all participants were invited to participate in a workshop in which single-platform enumeration methodology combined with dual-anchor T-cell gating strategy was addressed as the 'state-of-the-art' method. Fifty-five laboratories participated to this workshop (29); in case of poor performance, dedicated hands-on training was offered. The remaining laboratories participated in the regular spring 2001 EQA programme (send-out 11; see below).

Data processing and parameter classification

Prior to data processing, ambiguous data entries were corrected after review with the submitting participant. Each laboratory was assigned a unique number (ULN) for referral purposes. The absolute number of CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, and NK cells were assigned as response variables. The influence of the following 7 categorical variables on the outcomes of flow cytometric immunophenotyping assays (i.e., systematic differences ['bias'] and between-laboratory variability ['variation'] were investigated, and are summarized in Table 2 and discussed in detail below.

Table 2: Overview of categorical variables.

Variable	Categories
EQA send-out	1 – 20[a]
Unique Laboratory Number (ULN)	n.a.
Workshop 2001 participation	Yes
	No
Sample stabilization	No stabilization
	Stabilization
Gating strategy	FSC-SSC
	CD45-CD14
	CD45-SSC
	FSC-SSC-CD45-CD3
	FACSCount™ (BD Biosciences)
Platform methodology	Single
	Dual
Sample preparation	Lyse and Wash
	Lyse No Wash
	No Lyse No Wash
	Mononuclear Cells

Table 2: [a] Send-outs were numbered sequentially as a function of time.
n.a. = not applicable

EQA send-out.

Each send-out, from spring 1996 to autumn 2005 was chronologically assigned with a unique number (i.e., 1 to 20). In this way, we analyzed any effect of the EQA program on the variation of results as a function of time.

Participation to "workshop 2001."

Participation to this workshop was offered to all participants to this EQA scheme (see above) (29). We analyzed the effect of participation to this educational activity on the variation of results.

Sample stabilization.

Long-term stabilization of whole blood has been shown to reduce sample deterioration in EQA exercises for lymphocyte subset enumeration and CD34+ cell counting (22,23,28-32). We distinguished two categories: (i) no stabilization (n=75), and (ii) stabilization (i.e, short-term and long-term stabilization, n=5).

Gating strategies.

We distinguished five gating strategies; (i) FSC-SSC, (ii) CD45-CD14, (iii) CD45-SSC, (iv) FSC-SSC-CD45-CD3, and (v) FACSCount™ (BD Biosciences (San Jose, CA) (BD Biosciences)).

(i) *FSC-SSC gating.* In the late 1970s, implementation of single color analysis combined with dual light scatter for flow cytometric immunophenotyping was introduced. The combined FSC and SSC characteristics of leukocytes allowed the distinct clustering of lymphocyte, monocyte and granulocyte populations in a bivariate histogram (1). Once cell lineage specific markers were identified and multicolor (i.e., 3 or more) flow cytometry became available, FSC-SSC gating of lymphocytes became outdated.

(ii) *CD45-CD14 gating.* In the early 1990s, the combined analysis of immunophenotype and light scatter characteristics was introduced for gating on lymphocytes (33). By identifying the cell population of interest based on immunofluorescence, a light scatter window can then be drawn to include all (greater than or equal to 98%) of the lymphocytes. With this procedure, also known as 'backgating', recovery of the lymphocytes within the lymphocyte gate can be optimized. This information can also be used to identify cells other than lymphocytes within the light scatter gate. In this way it is possible to correct subsequent analyses since the reactivity of monoclonal antibodies on monocytes and granulocytes can be accounted for once cells other than lymphocytes have been identified as being within the acquisition gate (33).

(iii) *CD45-SSC gating.* Here, lymphocytes are identified by their CD45 and SSC characteristics (i.e., $CD45^{bright}$, SSC^{low}). CD45-SSC gates placed on lymphocytes should contain >95% lymphocytes (9,34). A possible disadvantage of this approach is the risk for exclusion of CD19+ B cells and NK cells from the lymphocyte gate; CD19+ B cells express slightly less CD45 than T cells, whilst NK cells have bright CD45 fluorescence but slightly higher SSC signals than the majority of lymphocytes) (34).

(iv) *FSC-SSC-CD45-CD3 gating.* With the introduction of fluorochromes such as peridinin chlorophyllin (PerCP) and allophycocyanin (APC), and tandem fluorochromes such as PE-Cy5 and PE-Texas Red, 3- and 4-color flow cytometric analyses became feasible. This development extended the possibilities for lymphocyte gating. With the "dual-anchor" approach (35), lymphocytes are selected first on the basis of bright CD45 expression and low SSC, followed by the selection of T cells on the basis of their CD3 positivity. Counterstaining of the T cells for CD4 and/or CD8 allows their further

characterization (34,35). These gating strategies were adopted subsequently in the CDC (36), NIAID-DAIDS (37,38), and British Committee for Standards in Haematology (BCSH) (39,40) guidelines.

(v) The *FACSCount*™ single-platform kit by BD Biosciences is based on counting beads (TruCOUNT™ tubes) and a no-lyse, no-wash (NLNW) sample preparation procedure. The kit utilizes two panels, i.e., CD4 PE/CD3 PE-Cy7 and CD8 PE/CD3 PE-Cy7 mAb mixtures. Using a CD3-FSC gate, most of the erythrocytes, platelets, monocytes, and granulocytes are excluded. A known number of reference beads included in each reagent tube functions as a fluorescence and quantitation standard for calculation of absolute CD3+, CD4+ and T-cell counts. Small numbers of laboratories used non-standard gating strategies or did not provide information on this point (see legend to Figure 1). These laboratories have been grouped , together with those using FACSCount™, as "Remainder" in Figure 1 (Panel A).

Platform methodology.

Absolute cell counts are traditionally assessed using a 'dual-platform' technique: (i) the flow cytometer provides the cell percentages as fractions of a denominator, i.e., WBC or lymphocytes, and (ii) the hematology analyzer provides the absolute WBC count together with a differential count, which must include the denominator. In the late 1990s 'single platform' techniques were introduced: the absolute cell counts are directly assessed on the flow cytometer in a precisely determined volume of blood sample. Single platform techniques can either be volumetric (41) or based on counting beads (42). The use of single platform techniques reportedly reduces between-laboratory variation in lymphocyte subset enumeration by eliminating the lymphocyte proportion from the hematology analyzer as a source of variation (22,43,44).

Sample preparation.

We distinguished four categories: (i) Lyse and Wash (LW); (ii) Lyse No Wash (LNW); (iii) No Lyse No Wash (NLNW); and iv) gradient separation of mononuclear cells (MNC). Variable cell losses, due to the use of different sample preparation techniques, would increase between-laboratory variation. Small numbers of laboratories did not provide information on this point (see legend to Figure 1). These laboratories have been grouped , together with those using NLNW, as "Remainder" in Figure 1 (Panel C).

Statistical analysis

For data processing and statistical analyses, the 'open source' program 'R' (http://www.r-project.org/) was used (Lucent Technologies, Murray Hill, NJ). First, a descriptive analysis of raw data of flow cytometric immunophenotyping was performed. This analysis revealed that the measurements had a low variability at low cell counts and

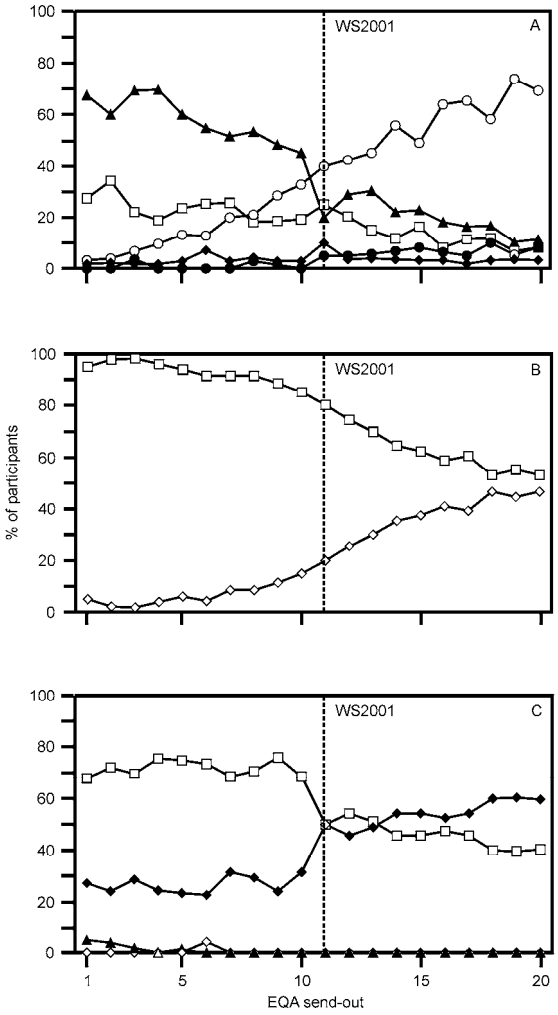

Figure 1: Change of usage patterns of methods over time. Panel A, gating strategies: □ = FSC-SSC, ▲ = CD45-CD14, ○ = CD45-SSC, ● = FSC-SSC-CD45-CD3, ◆ = Remainder (i.e., SSC-CD45-CD33-CD14; T/B lineage; FACScount™ or no information provided). Panel B, platform methodology: ◇ = Single platform, □ = Dual platform. Panel C, sample preparation: ◇ = Remainder (i.e, No Lyse No Wash or no information provided) □ = Lyse and Wash, ◆ = Lyse No Wash, ▲ = MNC. The vertical lines indicates the timing of the 2001 educational workshop (see Materials & Methods).

a high variability at high cell counts, which is often observed with cell enumeration data. As standard statistical techniques require an approximately constant variability over the whole cell count range, the data were logarithmically transformed. The logarithm is the transformation of choice for count data (45). To assess the effect of

multiple variables on the log-transformed flow cytometric immunophenotyping data we used robust multivariate regression (46). This approach is less sensitive to outliers than standard multivariate regression analysis. We then addressed two aspects of the quality of the log-transformed lymphocyte subset counts: bias (i.e., systematic differences) and variability (i.e., random differences) in separate analyses. Analysis of the mean of the log-transformed data revealed which variables caused systematic differences ('bias') in the mean lymphocyte subset counts. Subsequently, the bias was removed: the residuals of that analysis were used to investigate the variability of the lymphocyte subset counts. To this end the absolute values of these residuals (termed absolute error, which is related to the standard deviation) was used. As the distribution of the absolute errors was highly skewed, we have applied a Box-Cox transformation (47) in order to reduce this problem. A robust multivariate regression analysis of the Box-Cox transformed absolute errors was then performed to assess which variables affected the variability of lymphocyte subset counts. For each variable, the category with the most observations was chosen as benchmark. After log-transformation, the transformed data are shown in a linear scale with benchmark = 1, whilst after Box-Cox transformation, the transformed data is shown in a linear scale with benchmark = 0.

RESULTS

Methods used and change of usage patterns over time

From 1996 to 2005, 104 laboratories participated to 20 send-outs in our EQA scheme (50 to 71 participants per send-out). Data from 14 laboratories who submitted results to only 1 or 2 send-outs were excluded to avoid imbalance in the data. For analysis of list-mode data, various gating strategies have been used (Figure 1, panel A). The use of the "older" gating strategies declined over time: FSC-SSC (from 27% in 1996 to 8% of participants in 2005), and CD45-CD14 (from 68% in 1996 to 11% in 2005). In contrast, usage of methods recommended by guidelines (CDC, NIAID-DAIDS, and BCSH) increased: CD45-SSC (from 3% in 1996 to 69% in 2005), and dual-anchor gating (i.e., FSC-SSC-CD45-CD3) (from 0% in 1996 to 8% in 2005). After the 2001 workshop to which 55 laboratories participated, the use of CD45-SSC gating clearly increased, whilst that of lymphocyte backgating (CD45-CD14) decreased. To establish absolute counts (Figure 1, panel B), only 5% of the laboratories had adopted the single platform technique in 1996 versus 47% in 2005. The 2001 workshop did not lead to an accelerated implementation of single platform techniques. For sample preparation (Figure 1, panel C), the use of "lyse no wash" methods increased with time at the expense of that of "lyse and wash" methods, especially after the 2001 workshop.

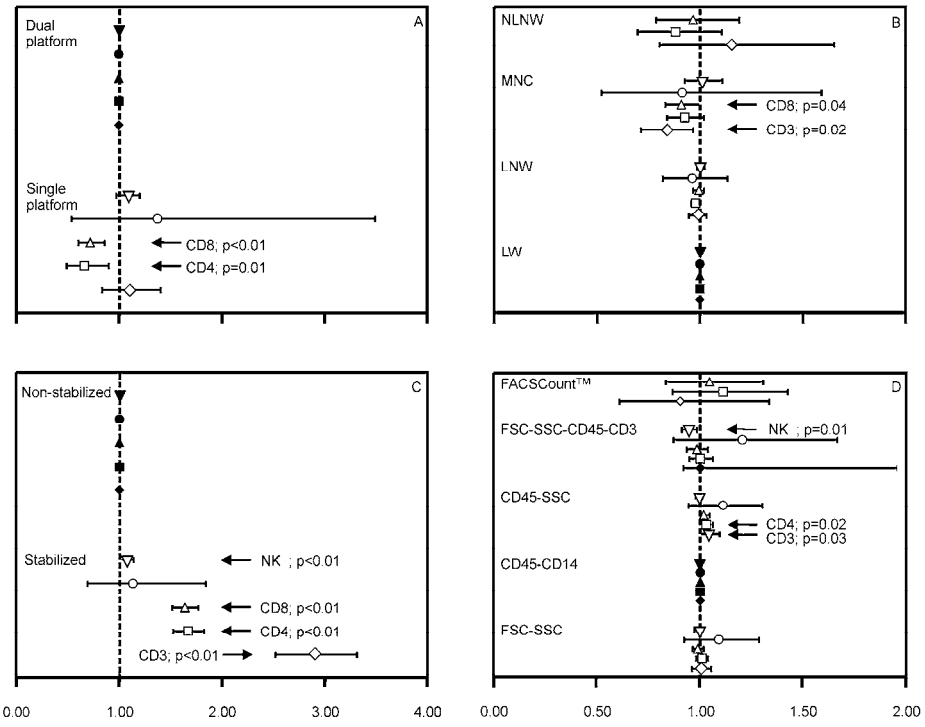

Figure 2: Factors significantly affecting the outcome of lymphocyte immunophenotyping. A: platform methodology. B: sample preparation. C: sample stabilization. D: gating strategy. The category with most observations was used as benchmark and assigned a factor value of 1.00. The graphs show the relative difference of the other categories related to the benchmark. Horizontal bars indicate 95% confidence intervals of the estimates of each factor value. *P*-values <0.05 are shown. Lymphocyte subsets are represented by the following symbols: ◇ = CD3+ T cells, □= CD4+ T cells, △ = CD8 T cells, ○ = B cells, ▽ = NK cells.

Eventually, all single platform users and a small proportion of dual platform users had adopted "lyse no wash" methods.

Factors affecting the outcomes of lymphocyte subset enumeration

We studied which of the 7 categorical variables (Table 2) significantly influenced the outcomes of lymphocyte subset enumeration. The effects of 5 variables were significant and are shown in Figures 2 and 3.

Platform methodology (Figure 2, panel A).

Most of the results were obtained using dual platform techniques. Therefore, this strategy was used as benchmark and assigned a factor value of 1 (see Materials

& Methods). In comparison, significantly lower results were obtained for CD4+ and CD8+ T cells using single platform as compared to dual platform techniques, whilst no significant bias was observed for CD3+ T cell, B-cell and NK-cell counts. The relatively few observations on B cells using single-platform techniques contributed to a wide confidence interval for this parameter.

Sample preparation (Figure 2, panel B).

Most results had been obtained using 'lyse and wash' methods. In comparison, lower counts were obtained for CD3+, CD4+ and CD8+ T cells after mononuclear cell isolation (significant for CD3+ and CD8+ T cells only). The results obtained with 'lyse no wash' and the small number of observations using 'no lyse no wash' methods were not significantly different from those obtained using "lyse and wash" methods.

Sample stabilization (Figure 2, panel C).

Most results had been obtained using non-stabilized samples. Significantly higher counts were obtained for all subsets except B cells using stabilized samples.

Gating strategy (Figure 2, panel D).

Most results had been obtained using the 'CD45-CD14' gating strategy, and relatively few using FACSCount™. The 'CD45-SSC' gating strategy yielded higher outcomes for CD3+, CD4+ and CD8+ T-cell counts (significant for CD3+ and CD4+ T cells only). Lower results were obtained for NK-cell counts using the 'FSC-SSC-CD45-CD3' gating strategy. The outcomes of the remaining 2 strategies, i.e., 'FSC-SSC' and FACSCount™, did not differ significantly from those of the 'CD45-CD14' gating strategy.

Laboratory (as defined by ULN; Figure 3).

Laboratory 27 was chosen as benchmark as it had no missing observations (Figure 3). The results of CD4+ T cells by 90 laboratories are shown in panel A. Six laboratories (i.e., 18, 20, 21, 34, 41 and 63) had factor values ~1.00, i.e., generated similar results as laboratory 27. The bias of CD4+ T-cell counts by 42 laboratories was positive in comparison to the benchmark laboratory (i.e., factor value >1.00); this bias was significant in 8 (indicated with arrows). The bias of 41 laboratories was negative, which reached significance in 2 (indicated with arrows). The patterns of deviation of CD3+ T-cell, CD8+ T-cell and B-cell counts were similar to those of CD4+ T cells (data not shown). The NK-cell counts, reported by 84 laboratories, stood out by having a pattern of bias that clearly differed from that of CD4+ T cells (panel B). Here, 5 laboratories (i.e., 11, 33, 47, 50 and 81) had factor values ~1.00, whilst the NK-cell counts of 17 were positively – but not significantly – biased, and those of 61 were negatively biased

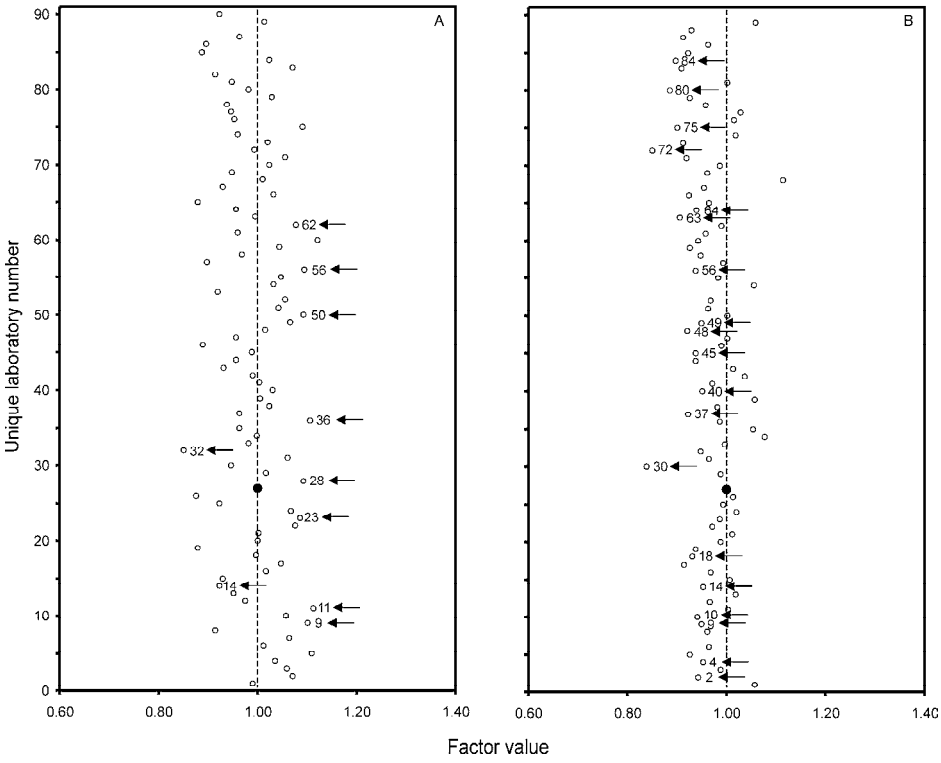

Figure 3: Outcomes of CD4+ T-cell (panel A) and NK-cell (panel B) enumerations by laboratory. Laboratory 27 had no missing data and was therefore chosen as 'benchmark' with factor value 1.00 (marked with a large, closed circle). The factor values reflect the relative differences between each individual laboratory and the benchmark laboratory. Laboratories with a significant bias relative to the benchmark laboratory are marked with arrows (i.e., *P*-values <0.05).

(significantly in 19 of them). Thus, benchmark laboratory 27 reported relatively high NK-cell counts in comparison to most of the other 83 laboratories.

The effects of the remaining categorical variables (i.e., EQA send-out and Workshop 2001 participation) on the outcomes of lymphocyte subset enumerations in terms of 'bias' were not significant.

Factors affecting the variability of lymphocyte subset counts

We studied which of the 7 categorical variables (Table 2) significantly influenced the variability of lymphocyte subset count measurements. The effects of 4 variables were significant as shown in Figures 4 to 6 and discussed below.

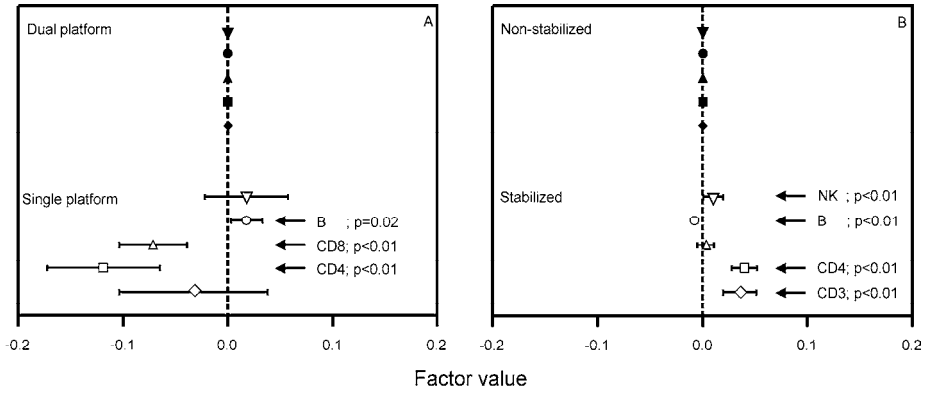

Figure 4: Factors significantly affecting the variability of lymphocyte immunophenotyping. Panel A: platform methodology. Panel B: sample stabilization. The category with most observations was used as benchmark and assigned factor value 0.00. The graphs show the relative differences between the other categories and the benchmark. Horizontal bars indicate 95% confidence intervals of the estimates of each factor value. *P*-values <0.05 are shown. Lymphocyte subsets are represented by the following symbols: ◇ = CD3+ T cells, □= CD4+ T cells, △ = CD8 T cells, ○ = B cells, ▽ = NK cells.

Platform methodology (Figure 4, panel A).

Most of the results were obtained using dual platform methodologies. Therefore, this strategy was used as benchmark and assigned a factor value of 0 (see Materials and Methods). The use of single-platform methods yielded significantly lower variability for CD4+ and CD8+ T-cell counts, but significantly higher variability for B-cell counts. The variability of CD3+ T cells and NK cells was similar for single and dual-platform methods.

Sample stabilization (Figure 4, panel B).

Most of the results were obtained using non-stabilized samples, which served as benchmark. Stabilized samples yielded significantly higher variability for CD3+ T-cell, CD4+ T-cell and NK-cell counts, but significantly lower variability for B-cell counts than non-stabilized samples. The variability observed for CD8+ T cells was similar for the 2 groups of samples.

Laboratory (as defined by ULN; Figure 5).

Laboratory 27 was chosen as reference as it had no missing observations. The results of CD4+ T cells by 90 laboratories are shown in Figure 5 (panel A). Four laboratories (i.e., 9, 16, 46 and 62) had factor values ~0.00, i.e., had similar variability in CD4+ T-cell counts as laboratory 27. The variability in CD4+ T-cell counts by 60 laboratories was larger in comparison to the benchmark laboratory (i.e., factor value >0); this variation was significantly larger in 10 (indicated with arrows). This variability was

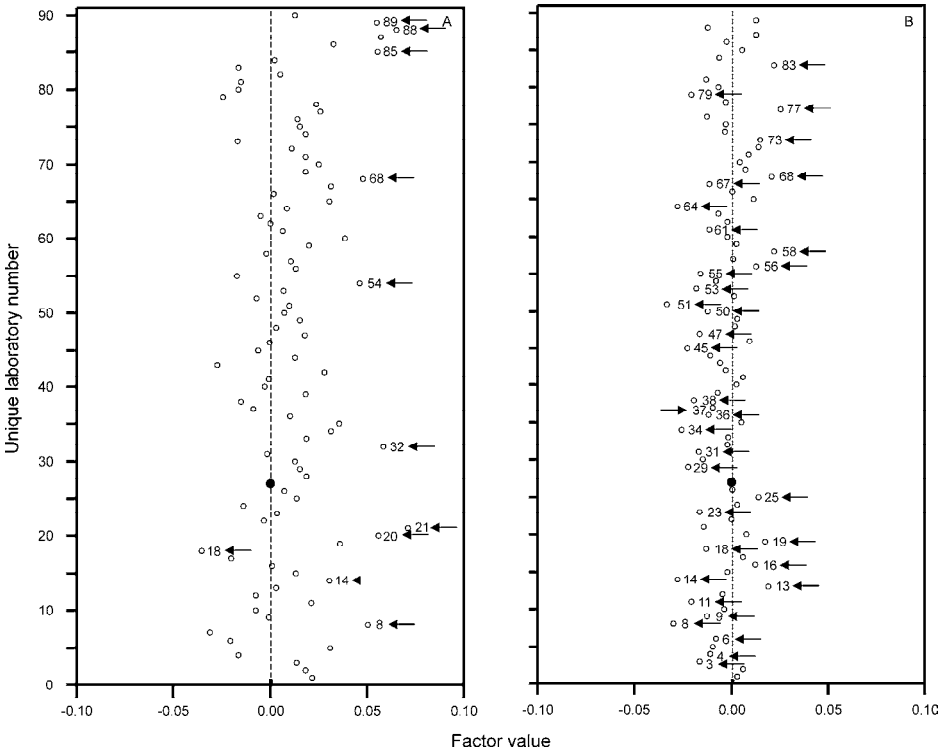

Figure 5: Variation of CD4+ T-cell (Panel A) and B-cell (Panel B) enumeration by laboratory. Laboratory 27 had no missing data and was therefore chosen as 'benchmark' with factor value 0.00 (marked with a larger, closed circle). The factor values reflect the relative difference between each laboratory and the benchmark laboratory. Laboratories with a significantly larger (factor value >0) or smaller (factor value <0) variation than the benchmark laboratory are marked with arrows (i.e., *P*-values <0.05).

smaller in 25 laboratories, which reached significance in 1 (indicated with arrow). The magnitudes of variation of CD3+ T-cell, CD8+ T-cell and NK-cell counts were similar to those of CD4+ T cell counts. The B-cell counts, reported by 86 laboratories, stood out by having a different pattern of variation (panel B). Here, 4 laboratories (i.e., 22, 26, 57 and 66) had variations similar to that of laboratory 27, whilst the variation was larger (i.e., factor value >0) in 31 laboratories (significantly in 10), and smaller in 50 (significantly in 25). Thus, benchmark laboratory 27 had a relatively large variation in B-cell counts in comparison to the other 85 laboratories.

EQA send-out number (Figure 6).

The test variations of the 5 lymphocyte subset counts as a function of send-out number (1 to 20) is set out in Figure 6. The first send-out was taken as benchmark. In general,

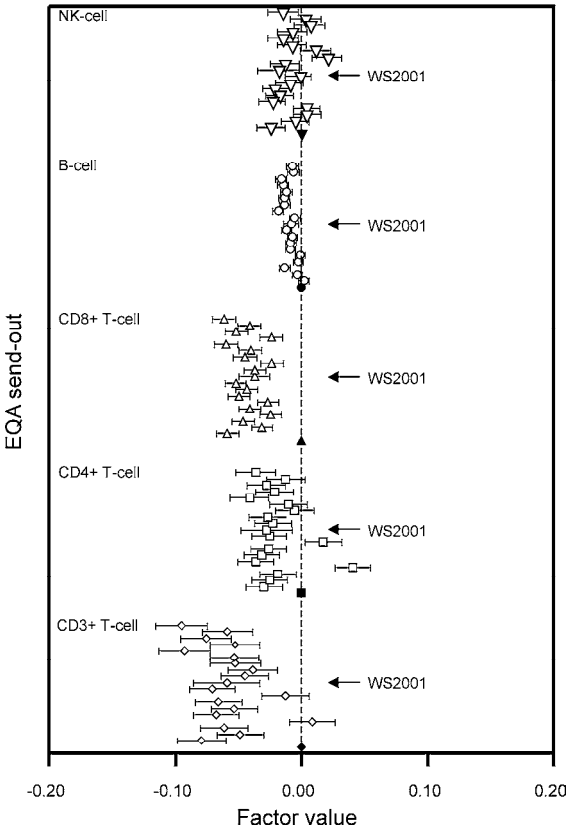

Figure 6: Analysis of the variability of lymphocyte subset enumeration by send-out. For each subset, the first send-out is shown at the bottom of each series, followed by the other 19 ranked in ascending order as a function of time. Lymphocyte subsets are represented by the following symbols: ◇ = CD3+ T cells, □ = CD4+ T cells, △ = CD8+ T cells, ○ = B cells, ▽ = NK cells. For each lymphocyte subset, the first send-out was taken as 'benchmark' and assigned factor value 0.00. The horizontal lines show the 95% confidence intervals of the estimation of each factor value.

the variation for all 5 subsets was significantly smaller in the subsequent 19 send-outs with a few exceptions, i.e., send-outs 5 and 9 for CD4+ T-cell counts, and send-outs 13 and 14 for NK-cell counts. The standardization workshop (55 participating laboratories) was held concurrently with send-out 11, to which the remaining laboratories participated (indicated with arrows in Figure 6). The variability of B-cell counts in the 9 "post-workshop" send-outs was significantly lower than in the 11 send-outs that were held prior to or concurrent with the workshop (P = 0.01 using the Kruskal-Wallis test). For the other lymphocyte subsets, differences between test variations prior to and after the workshop were not significant.

DISCUSSION

Here, we review our 10-year experience with the biannual external quality assessment (EQA) programme for lymphocyte subset enumeration in the Benelux countries. We analyzed results from nearly 23,000 assays reported by more than 100 laboratories in the context of methodological information provided in questionnaires with each send-out. The main characteristics of this EQA program were: (i) use of abnormal samples in 69% of cases; (ii) use of fresh samples in 94% of cases; (iii) debriefing with personalized recommendations after each send-out, plus annual plenary meetings in which the results were reviewed and discussed with the participants. The purpose of providing fresh, "pathological" samples was to mimic real-time situations in the daily practice of the clinical laboratory. Half way this program, an educational workshop was organized in which 55 participants were trained in the use of what was considered as 'state-of-the-art' method (single-platform method with dual-anchor gating strategy) (29). In the current study, we addressed 3 aspects: (i) change of usage of methods over time; (ii) the influence of methodological variables on the outcome of lymphocyte subset enumeration (i.e., bias); and (iii) the influence of methodological variables on the variation of lymphocyte subset enumeration. We also analyzed whether or not this variation declined with time.

Our recommendations provided with the debriefings and the plenary meetings reflected international recommendations (9,36-40,48). As a result, the use of 'CD45-SSC' gating increased at the expense of 'CD45-14 backgating', and 'lyse-no-wash' techniques were implemented by the majority of participants instead of 'lyse & wash' techniques. In spite of our recommendations, 'dual-platform' counting techniques remained the preferred approach by the majority of participants. Discussions at the plenary meetings revealed that single-platform techniques were considered to be costly (increased expenses associated with counting beads) and cumbersome (more complicated requirements associated with 'reverse pipetting') in comparison to dual-platform techniques.

A negative bias for CD4+ and CD8+ T-cell counts, but not for CD3+ T cells, B cells or NK cells was associated with the use of single-platform counting techniques. Similar observations with respect to CD4+ and CD8+ T-cell counts have been made by Reimann *et al* (44). Other studies have identified the lymphocyte count derived from the hematology analyzer as the main source of bias in CD4+ T-cell counts (49). It should be noted that conditions in our EQA scheme were suboptimal for dual-platform techniques. Most samples were non-stabilized and ~24h old when tested by the participants, whilst guidelines require that absolute lymphocyte counts by hematology analyzer be performed within 6h after venipuncture (36). Our review of used methodologies showed that most laboratories used the leukocyte differential to calculate

absolute lymphocyte counts, followed by multiplication with % CD4+ cells (fraction of lymphocytes). However, it is recommended for suboptimal samples to use only the absolute leukocyte count from the hematology analyzer, multiplied by % CD4+ cells (fraction of leukocytes) (50). This usage pattern did not change in spite of our repeated recommendations (not shown). We suggest that overestimation of the % lymphocytes using dual-platform techniques may have contributed to the observed bias in CD4+ and CD8+ T-cell counts. However, the lack of such bias for CD3+ T-cell, B-cell and NK-cell counts remains unexplained.

The use of MNC separation has been discouraged because of the risk for selective cell loss (51), and has generally been abandoned in our study after the first send-outs. It seems safe to suggest that the bias towards lower CD3+, CD4+ and CD8+ T-cell counts associated with MNC isolation is due to such losses.

Strikingly, stabilization showed a bias towards high CD3+, CD4+, CD8+ T-cell counts as well as high NK-cell counts. This result may be explained by some decay of the fresh blood samples during shipment and storage prior to testing. The absence of this bias for B-cell counts would be explained by the observation that loss of CD19 intensity occurs upon stabilization (30,31).

As for gating, the 'CD45-SSC' strategy stood out by a positive bias for CD3+ and CD4+ T-cell counts relative to the 'CD45-14 backgating' strategy. Currently, the view is widely held that 'CD45-SSC' gating of lymphocytes is more robust than 'CD45-14 backgating' (34,37,39); the risk of the latter method is that a too restricted FSC-SSC gate is set on lymphocytes in order to avoid contamination by monocytes, which then would result in an underestimation of the % CD3+ or CD4+ T cells (52). Furthermore, the 'dual-anchor' gating strategy was associated with a negative bias towards NK cells. This method defines a 3-dimensional gate using FSC, SSC and CD45, and has been optimized for single-platform T-cell subset enumeration based on the expression of CD3 by T cells (31). However, a disadvantage of this approach may be the selective loss of CD3 negative lymphocytes due to differences between CD3+ and CD3- cells in CD45 expression and light scatter characteristics. This situation may have contributed to underestimation of NK-cell counts.

Some parameters also influenced the variability of lymphocyte subset enumeration. The variability of CD4+ and CD8+ T-cell counts was smaller with the use of single-platform assays than when dual-platform assays were used. This observation confirms previous studies (22,29,38,43,44). Single-platform assays bypass the 'denominator issue', i.e., the need to express the percentage of lymphocytes as a proportion of either leukocytes, total nucleated cells or total events exceeding the FSC threshold. Also, single platform assays avoid the variability arising from hematology analyzers used to enumerate total nucleated cells or leukocytes (49). We do not have an expla-

nation for the larger variation of B-cell enumerations in single-platform assays in our study, but our data were concordant with various other studies (27,53).

The variability of CD3+ and CD4+ T-cell counts as well as NK-cell counts was larger when stabilized samples were tested, whilst the variability of B-cell counts was smaller in comparison to non-stabilized samples. In contrast, when using between-laboratory CV as parameter, the variability of CD3+, CD4+ and CD8+ T-cell counts was smaller when stabilized samples were tested as compared to fresh samples (28). In line with the results of a similar comparative study (23), a possible explanation of the larger variability with stabilized samples in our study is that these samples were only a minority of test samples (i.e., 5 of 80) and that the participants were not familiar with the (slightly) different light scatter characteristics and fluorescence patterns of stabilized specimens.

Several individual laboratories stood out by significant positive or negative bias and/or higher or lower variability relative to the benchmark laboratory. As this result was obtained in a multivariate analysis, such bias and variability must have been due to other factors than the categorical variables listed in Table 2. Due to the limitations imposed by the study design - for example, no central review of list mode data has been performed in order to find explanations for outlying results -, it was not possible to trace the causes of large bias or variability for individual laboratories.

Last but not least, we analyzed whether or not the implementation of this EQA scheme was effective in reducing test variation as a function of time. The first send-out was exceptional with a high variability of all subset counts, but especially of the CD3+, CD4+ and CD8+ T cells. Thereafter, test variation declined with occasional exceptions. Test variation of B-cell counts declined after the 2001 workshop, but this exercise was specifically aimed to standardize T-cell subset enumeration (29). Nevertheless, inspection of the variation of CD3+, CD4+ and CD8+ T-cell counts as a function of time reveals a trend towards lower variation (Figure 6). Therefore, this EQA exercise may indeed have resulted in some reduction of test variability, although it should be realized that the participants standardized their techniques only partially over time. They did so with respect to sample preparation and gating strategies, but not with absolute counting techniques. Only when participants are fully committed to standardize their techniques, such as in the Canadian Clinical Trials Network for HIV/AIDS Therapies (35), can significant reductions in between-laboratory variation of lymphocyte subset enumeration be achieved.

Whilst participation to EQA of lymphocyte immunophenotyping has been on a voluntary basis in the Benelux countries, results of EQA exercises are increasingly being used for laboratory accreditation. When results of EQA exercises will have financial consequences - e.g., the need for accreditation status to claim reimbursements by health care insurance - it becomes imperative that such programs themselves meet

rigorous quality demands. Such demands include documentation of the quality of distributed samples and the use of validated procedures for evaluating results. Meeting these demands will be a challenge for EQA of lymphocyte subset enumeration in the immediate future.

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J.C. Kluin-Nelemans (LUMC, Leiden); P.A. Kuiper-Kramer (Hospital 'Isala Klinieken', Zwolle); P.C. Limburg (UMCG, Groningen); B.E.M. v.d. Linden-Schrever (SKION, Den Haag); E. van Lochem (Erasmus MC, Rotterdam); C.L. Lodowika (Alysis Zorggroep, Arnhem); B. Lunter (Twente University, Enschede). R. Malfait (Hospital 'Middelheim', Antwerpen (B)); A. Martens (Hospital 'Twente', Almelo); A.M. Mazzon (UCL Ecole de Sante Publique, Brussel (B)); P. Meeus (Hospital 'Onze Lieve Vrouwe Kliniek', Aalst (B); M. de Metz (Hospital 'Canisius Wilhelmina', Nijmegen); X. Meyer-Stillemans (Laboratory 'St. Elisabeth', Namur (B)); E. Moreau (Hospital 'Heilig Hart', Roeselare (B)); E. Mul (Sanquin Diagnostics, Amsterdam); [B023] A.B. Mulder (Hospital 'Jeroen Bosch', Den Bosch); W.J. Nooijen (Hospital 'Antonie van Leeuwenhoek', Amsterdam); Th. Out (AMC, Amsterdam); D.S. Park (Hospital 'Slotervaart', Amsterdam); J. Pattinama (Hospital 'St. Franciscus Gasthuis', Rotterdam); J. Phillipé (UZG, Gent (B)); O. Pradier (Hospital 'Erasme', Brussel (B)); F.W.M.B. Preijers (UMC St. Radbout, Nijmegen); H.J. Puts (Gelre Hospitals, Apeldoorn); S. Rensink Matter (Hospital 'St. Lucas Andreas, Amsterdam); F. Reymer (LUMC, Leiden)); G.T. Rijkers (Hospital 'Wilhelmina kinderziekenhuis', Utrecht); L.G. Rijks (Hospital 'Vlietland', Vlaardingen); M. Rijpert-van Son (Hospital 'Tweesteden', Tilburg); M. Roos (Sanquin Diagnostics, Amsterdam); K.J. Roozendaal (Hospital 'Onze Lieve Vrouwegasthuis', Amsterdam); J.L. Rummens (Hospital 'Virga Jesse', Hasselt (B)); L.J.M. Sabbe (Laboratory 'Zeeland', Goes); P. de Schouwer (Hospital 'Stuivenberg', Antwerpen (B)); W. Slieker (MC Alkmaar, Alkmaar); R.J. Slingerland (Hospital 'Isala Klinieken', Zwolle); J.W. Smit (UMCG, Groningen); M. van Tol (UMCG, Groningen); T.A.M. Trienekens (VieCuri, Venlo); H.L. Vader (Maxima Medical Center-Veldhoven, Veldhoven); L.M.B. Vaessen (Erasmus MC, Rotterdam); A. Vanhouteghem (Hospital 'Middelares', Gent (B)); W. Veenendaal (Hospital 'Leyenburg', Den Haag); J. Verschaeren (Clinical laboratory, Antwerpen (B)); M. de Waele (AZ-VUB, Brussel); G. Wallef (Hospital 'de Jolimont', Haine-Saint-Paul (B)); J.W.J. van Wersch (Hospital 'Atrium Heerlen', Heerlen); F.L.A. Willekens (Alysis Zorggroep, Arnhem); A. Wolthuis (Clinical Laboratory, Leeuwarden).

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8

GENERAL DISCUSSION

8.1 FLOW CYTOMETRIC HLA-B27 SCREENING

8.1.1 Educational aspects

The results of the Benelux EQA scheme for flow cytometric HLA-B27 screening offered the unique opportunity to analyze the pitfalls of flow cytometric HLA-B27 screening in the setting of a multicenter study (Chapter 2). In this retrospective analysis cross-reactivity of HLA-B27 mAb was observed and revealed as a major pitfall in flow cytometric HLA-B27 screening. While only frequent HLA-B27 subtypes B*2705 and B*2702 were send out, no additional information regarding the variable reactivity of HLA-B27 mAb with the different HLA-B27 alleles (as defined by DNA typing and summarized in Table 2 of the General Introduction) is derived. In order to optimally advice laboratories performing flow cytometric HLA-B27 screening, we performed a comprehensive analysis of the cross-reactivity patterns from commercially available HLA-B27 screening reagents, based on 3 HLA-B27 mAb clones, i.e., ABC-m3 (Serotec, Immunotech and Silenus), GS145.2 (BD Biosciences) and FD705 (OneLambda) (Chapter 3).

For the ABC-m3-based reagents, we observe strong cross-reactivities with HLA-B27 negative lymphocytes expressing HLA-B7, B22, B37 and B42, whilst weak cross-reactivities were observed with HLA-B12, B13, B16, B17, B40, B41, B47 and B48. These results confirm and extend previous observations, specifically with respect to HLA-B7 (1-8), B12 and B37 (1,2). For the GS145.2 mAb, we observe strong cross-reactivities with HLA-B7, B16, B37, B42 and B48, and weak cross-reactivities with HLA-B13, B17, B22, B40 and B47. These results confirm and extend previous observations, specifically with respect to HLA-B7 (1,3,9,10), B16 and B37 (1,9). However, we did not observe the previously reported cross-reactivity with HLA-B12 (1). For the FD705 mAb, we observe strong cross-reactivities with HLA-B17, B37 and B47, and weak cross-reactivities with HLA-B12, B13, B16 and B48. These results confirm and extend previous observations, specifically with respect to HLA-B7 (3,4,11) and HLA-B12, B17 and B37. On the basis of the results as discussed above, we conclude that FD705 is not, in contrast with other studies (3,4,11, monospecific for HLA-B27) but shows the lowest degree of cross-reactivity with other HLA-B antigens in comparison with the ABC-m3 and GS145.2 mAb. A disadvantage of FD705 is that it not bound to the protein encoded by the B*2708 allele (12 and Chapter 3). Based on the knowledge of cross-reactivity, we do not send out samples that would be the normal workload of the participants, but we deliberately target samples with cross-reacting HLA-B antigens that could cause errors.

8.1.2 Trends toward improvement

None of the 5 HLA-B27 screening reagents tested in our study (Chapter 3), reacted monospecific with the HLA-B27 antigen. Importantly, in HLA-B27 negative, HLA-B

heterozygous donors, the use of 2 HLA-B27 mAb to obtain a definite HLA-B27 typing result by flow cytometry would have avoided any false-positive conclusion on our extensive cell panel. In HLA-B27 negative, HLA-B homozygous donors, any combination of ABC-m3, GS145.2 and FD705 would have classified a single HLA-B homozygous donor as HLA-B27 positive. So, the strategy using 2 HLA-B27 mAb is not absolutely error-proof, but conflicting results would have been reduced to a minimum by a combination of the FD705 mAb and the GS145.2-based screening kit. Therefore, we recommend the combination of the FD705 mAb and the GS145.2-based HLA-B27 kit as the most robust approach for flow cytometric HLA-B27 screening (Chapter 4).

In addition, as for many clinical laboratories the availability of fresh HLA-typed blood samples as control cells is cumbersome, we analyzed the feasibility of long-term stabilized blood samples (as performed by UK NEQAS for Leucocyte Immunophenotyping) (13-20) i) as control cells (i.e., HLA-B27 positive and negative control cells for each batch of assays), ii) for the validation of reagents used in the procedure of flow cytometric HLA-B27 screening, and additionally iii) as test samples for EQA of flow cytometric HLA-B27 screening (Chapter 5).

In our study, we have shown that the use of long-term stabilized samples with positive and negative serological reactivities is feasible as positive and negative test control cells for each batch of tests with a shelf life up to 12 months. For reagent validation, cells expressing HLA-B antigens belonging to serological cross-reacting groups are essential to verify the specificity of mAb. In addition, the FL1 mean fluorescence intensity (MFI) of the long-term stabilized samples was, in general, lower than those of fresh cells. Therefore, long-term stabilized blood samples do not meet the strict requirements for reagent validation purposes, and fresh samples should be used for this purpose. For EQA purposes, long-term stabilized samples are feasible with the same caveats as for the test control cells and procedure controls.

In general, extensive quality control of stabilized samples is necessary before distribution to the laboratories to avoid problems with poor FSC-SSC characteristics and extensive decrease of the FL1 MFI obtained with HLA-B27 flow cytometric typing reagents.

8.1.3 Future developments in flow cytometric HLA-B27 typing

Nowadays, about 85% of the participants of the Benelux EQA scheme for HLA-B27 screening is using flow cytometry. However, despite of the improvements as discussed before, flow cytometric HLA-B27 screening cannot avoid any false-positive or, in lesser extend, false-negative conclusions. Therefore, positive or conflicting results between any combinations of HLA-B27 mAb would require further testing by a reference technique. For HLA-B27 HLA-typing, DNA-based typing methods now are available (11,21). Another big advance of DNA-based typing methods is the possibility to assign the

HLA-B27 subtypes (22). This may be of clinical relevance, as the presence of ankylosing spondilytis (AS) and related spondyloarthropathy (SpA) has been documented in individuals possessing the relatively more common subtypes (e.g., B*2705, B*2704, B*2702, and B*2707). In addition, other subtypes (e.g., B*2706 and B*2709) may carry to a lesser degree of disease susceptibility in AS (reviewed in 79). As molecular biology will become more commonly applied and its costs relatively decrease, DNA-based HLA-B27 (sub)typing may be common practice, with the surplus value of proper disease risk classification in, for example, AS and SpA. Tissue typing laboratories accredited by the American Society for Histocompatibility and Immunogenetics or the European Federation for Immunogenetics play an indispensable role in such.

8.2 FLOW CYTOMETRIC CD34+ CELL ENUMERATION

8.2.1 Educational aspects

In the past, several EQA schemes for CD34+ cell enumeration have been set up and workshops have been organized to train and implement standardized protocols for clinical laboratories performing flow cytometric CD34+ cell enumeration (23-34). Meanwhile, widely varying inter-laboratory coefficients of variation (CVs) have been reported. An overview of inter-laboratory variation in various multicenter trials and external quality schemes is shown in Table 1. To explain the widely variation in CD34+ cell enumeration between laboratories, the results of the Benelux EQA scheme for flow cytometric CD34+ cell enumeration have been analyzed using robust multivariate regression. This approach is relatively insensitive to outliers and is used to assess the effect of methodological aspects of CD34+ cell enumeration on the bias and variability (Chapter 6).

8.2.2 Trends toward improvement

Five variables had significant effects on the outcome of flow cytometric CD34+ cell enumeration: (i) gating strategy; (ii) labeling of CD34 mAb; (iii) flow cytometer type, (iv) sample preparation; and (v) individual laboratories.

First, for the gating strategies, the Milan and Stem-KIT™ approaches generated significantly lower CD34+ cell counts in comparison to other approaches. With the Milan approach, utilizing the selection of leukocytes based on FSC and SSC and only counting bright CD34+ cells, these findings can be explained. The exclusion of debris, red blood cells, and platelets based on light scatter is not complete. Also dim CD34+ cells are not included. We extensively examined why the Stem-KIT™ approach generated lower CD34+ counts. Certainly, the Stem-KIT™ approach is similar to that of the

Table 1: External quality assessment and trials in flow cytometric CD34+ cell enumeration in multicenter studies

Study[1,2]	# participants	Type of specimens[3]	Range of CD34+ cells[5]	# Specimens distributed	Assay method used	Inter-laboratory CV (range)
RMS-CFCG (23)	15	fPB, fBM, fCB	0.1-19.3[6]	28	Various	62-100
ABS-SG (24)	20	fPBSC	10	1	Various	29
SIHON I (25)	29-34	fPB, fPBSC	1.2-3.9[6]	2 pairs[8]	Various	34-106
	24-26	fPB, fPBSC	1.1-3.7[6]	2 pairs[8]	SIHON	18-30
AOR-SCG (26)	11	fPBSC	0.4-5.0	2	Various	25-48
Canadian (27)	14-19	fCB	15-148	4	Various	30-134
INSTAND (28)	44	StemTROL™[4]	9-16	2	Various	56-89
UK-NEQAS I (29)	64-79	sPB	8-158	6	Various	26-76
	80-84	sPB, sCB	14-63	2 pairs[9]	Various	14-43
BEST (30)	12	fPB	5-200	29	ProCOUNT™	8-63
	12	StemTROL™[4]	10-200	5	ProCOUNT™	14-36
UK-NEQAS II (31)	69-85	sPB	11-95	9	Various, dual pltf[10]	19-44
	8-14	sPB	11-95	9	Various, single pltf[10]	3-37
EWGCCA (32)	24	sPB	150-210	3	ISHAGE single pltf[10]	10-23
CAD-CBMTG (33)	6	fPBSC	0.1-32.8[7]	25	Various	14-174
SIHON II (34)	9	sPB	35-60	3	ISHAGE single pltf[10]	9-28
	27	sPB	35-60	3	Various dual pltf[10]	17-31
UK-NEQAS III (34)	23	sPB	35-60	3	Various single pltf[10]	9-14
	92	sPB	35-60	3	Various dual pltf[10]	21-23

Table 1: [1] Literature citations are numbered and listed between brackets; the numbers correspond to those used in the Literature Cited section; [2] Abbreviations: RMS-CFCG, Royal Microscopical Society Clinical Flow Cytometry Group; ABS-SG, Australasian BMT Scientists Study Group; SIHON, Stichting Immunofenotypering Hematologische Oncologie Nederland; AOR-SCG, American Oncology Resources Stem Cell Group; INSTAND, Institute for Laboratory Standardization and Documentation; UK-NEQAS, United Kingdom National External Quality Assessment Schemes; BEST, Biomedical Excellence for Safer Transfusion Working Party; EWGCCA, European Working Group on Clinical Cell Analysis; CAD-CBMTG, Canadian Apheresis Group and Canadian Bone Marrow Transplant Group; [3] Abbreviations: f, fresh; s, stabilized; BM, bone marrow; CB, cord blood; PB, peripheral blood; PBSC, peripheral blood stem cell collection; [4] StemTROL™: stabilized StemTROL™ (Beckman-Coulter/Immuno-tech, Marseille, France) were spiked into fresh peripheral blood; [5] Number of CD34+ cells per µl; [6] Percentage of CD34+ cells within leukocyte deno-minator; [7] Number of CD34+ cells x 10^6 per kg body weight of the recipient; [8] Paired PB and PBSC of one patient; [9] One of each pair was issued with a known number CD34+ cells, determined by the coordinating laboratory; [10] Abbreviation: pltf, platform

ISHAGE protocol so similar CD34+ cell counts would have been expected with both approaches. Therefore, data analysis files of the 4 participants involved were requested and checked. No inconsistencies were found. In addition, the 4 participants did not have other methodological features in common, such as type of flow cytometer and data analysis software, except using the Stem-KIT™ reagents. Thus, we do not have an explanation for the relatively low CD34+ cell counts observed with the Stem-KIT™ gating strategy.

Second, for labeling of CD34 mAb, the FITC conjugates yielded lower absolute CD34+ cell counts than the PE conjugates. This difference may be explained by the fact that PE emits stronger fluorescent signals and therefore allows better separation between positive and negative populations than does FITC.

Third, with the FACScalibur™ and Cytoron™ flow cytometers, higher CD34+ cell counts were obtained in comparison to other instruments. As a volumetric instrument, the CytoronAbsolute™ is less suitable for enumeration of samples with low CD34+ cell counts as it can only acquire and analyze a limited volume of sample and cells in a single run (35). The optical systems of FACScalibur™ instruments and FACScan™ instruments are highly similar. FACScalibur™ instruments are generally more recent than FACScan™ instruments, which may result in a higher sensitivity for dim CD34+ populations and may explain the systematically higher CD34+ cell counts observed with FACScalibur™ instruments as compared to FACScan™ instruments.

Fourth, the LNW technique generated slightly higher absolute CD34+ cell counts as compared to the other sample preparation techniques. Washing in the LW technique may have resulted in (selective) cell losses, in the NLNW technique an underestimation of CD34+ cell counts due to the large proportions of red cells in the unlysed samples may occur, and the use of MNC separation was discouraged because of the risk of selective cell loss (36).

Two variables had significant effects on the variability of flow cytometric CD34+ cell enumeration: (i) platform methodology; and (ii) individual laboratories.

First, our analysis confirms the studies by Barnett *et al*, 2000; Chang *et al*, 2004 and Gratama *et al*, 2003, which showed that dual-platform techniques are associated with higher variability than single platform techniques (29,32,34,37). Furthermore, our analysis shows that the implementation of single platform methodology is highly effective in reducing between-laboratory variability of this assay.

An analysis of the outcome and variability of CD34+ cell counts as a function of individual laboratories revealed that some laboratories stood out by obtaining relatively high CD34+ cell counts, and others by obtaining a relatively large variation in CD34+ cell counting results. As the participants had not been requested to keep flow cytometric list mode data on file for this purpose, it was not possible to trace the causes of bias and variability for this study.

As clearly indicated by the results of our study, the state of the art methodology for flow cytometric CD34+ cell enumeration should be a single platform assay with sequential Boolean gating, as proposed by the ISHAGE (38). At the moment, this approach is most robust and shows the lowest variability between laboratories. Importantly, the variability of CD34+ cell enumeration also declined significantly with time. As most send-outs with a relatively small variability in CD34+ cell counts fell after the organization of a practical workshop in which the single platform ISHAGE protocol was introduced (35), it seems reasonable to state that this exercise has been effective in reducing between-laboratory variability.

8.2.3 Future developments in flow cytometric CD34+ cell enumeration

Advanced studies in grafts of various stem cell sources (e.g., bone marrow [BM], peripheral blood [PB] and cord blood [CB]) and correlation with engraftment kinetics in such cases may prove to be important for poorly mobilizing patients and to optimize utilization of clinical resources in the setting of human stem cell (HSC) transplants. In this way, flow cytometric CD34+ subset enumeration is indispensable. For example, several clinical trials using highly purified lin-CD34+CD90+ populations have shown rapid and sustained engraftment when as few as 8x105 selected CD34+CD90+ cells per kg recipient body weight were given to patients with breast cancer (39), non-Hodgkins lymphoma (40) and multiple myeloma (41). Also, the CD34+CD90+ subset best predicted platelet recovery during the first 12 months after transplant (42). Furthermore, the distribution of CD34+ subsets in BM, G-CSF mobilized PB and CB is different. Where the proportions of CD34+, CD38- and CD34+, HLA-DR- cells do not differ among these compartments, the co-expression of more mature CD34+ cells with CD13 and CD33 is higher in PB and CB compared with those of BM. In contrast, the proportion of CD34+, CD19+ cells is higher in BM. The proportion of CD34+, CD3+ cells is comparable in all three compartments (43). As myeloid progenitors are associated with early engraftment, flow cytometric CD34+ subset enumeration may play a role in predicting engraftment kinetics.

In addition, detailed phenotyping of CD34+ blast cells (BCs) can be important for clinical decision making in hematological malignancies (44,45). For example in patients with MDS and acute leukemia transformed from MDS, flow cytometry revealed that a high proportion of the BCs showed an immunophenotype of committed myeloid precursors (CD34+, CD38+, HLA-DR+, CD13+, and CD33+). However, markers for myeloid cell maturation (CD10 and CD15) were more prevalent on BCs from low-risk MDS (e.g., refractory anemia), whereas markers for myeloid cell immaturity (CD7 and CD117) were more prevalent on BCs from high risk MDS (e.g., chronic myelomonocytic leukemia and RA with excess blasts) and AL-MDS (44).

Above mentioned applications require flow cytometric techniques that are capable to accurately monitor CD34+ cells and subsets. Based on our results, the single platform ISHAGE protocol using a PE-labeled CD34 class III mAb allows a direct, robust and reproducible quantification of hematopoietic stem cells in any source (e.g., apheresis product, PB, BM, and CB). International standardization of the flow cytometric techniques used should improve the between-laboratory variation of the enumerations obtained, so that inter-institutional results become more meaningfully comparable.

8.3 FLOW CYTOMETRIC LYMPHOCYTE SUBSET ENUMERATION

8.3.1 Educational aspects

Large variations between results of individual centers was observed in various multicenter studies (16,19,31,46-59). An overview of inter-laboratory variation in various multicenter trials and external quality schemes is shown in Table 2. To explain the widely variation in lymphocyte subset enumeration between laboratories, the results of the Benelux EQA scheme for flow cytometric lymphocyte subset enumeration have been analyzed using robust multivariate regression. This approach is relatively insensitive to outliers and is used to assess the effect of methodological aspects of lymphocyte subset enumeration on the bias and variability (Chapter 7).

8.3.2 Trends toward improvement

Five variables had significant effects on the outcome of flow cytometric lymphocyte subset enumeration: (i) platform methodology; (ii) sample preparation; (iii) sample stabilization; (iv) gating strategy, and (v) individual laboratories.

First, for platform methodology, a significant negative bias for CD4+ and CD8+ T-cell counts, but not for CD3+ T cells, B cells or NK cells was associated with the use of single-platform counting techniques. Overestimation of the % lymphocytes using dual-platform techniques may have contributed to this bias in CD4+ and CD8+ T cell counts. Whilst guidelines require that absolute lymphocyte counts by hematology analyzer be performed within 6h after venipuncture (60), most samples were non-stabilized and ~24h old when tested by the participants. Furthermore, most laboratories used the leukocyte differential to calculate absolute lymphocyte counts, followed by multiplication with % CD4+ cells (fraction of lymphocytes). However, it is recommended for sub optimal samples to use only the absolute leukocyte count from the hematology analyzer, multiplied by % CD4+ cells (fraction of leukocytes) (61).

Second, for sample preparation, the use of MNC separation has been discouraged because of the risk for selective cell loss (62). We suggest that the bias towards lower

Table 2: External quality assessment and trials in flow cytometric lymphocyte subset enumeration in multicenter studies

Study[1,2]	# participants	Type of specimens[3]	Range of CD4+ cells[4]	# Specimens distributed	Assay method used	Inter-laboratory CV (range)[8]
NIH/NIAID (46)	80-100	fPB	29-58[5]	3	Various	5-71
ACTG (47)	20-46	fPB	14-77[5]	10 pairs[6]	Various	5-56
CAP-FCS (48)	169-222	fPB, fPBMC	24.7-59.0[5]	8	Various	7.8-55.7
NIH/MACS (49)	4	fPB	4.5-57.9[5]	78	CD45-CD14	1.6-17
Etalonorme (50)	62-129	fPB,sPBMC	unknown	7	Various	6-38
GIC (51)	306	sPBMC	unknown	1	Various	8-48
CEQUAL (52)	33-35	fPB, sfPBMC	15-65[5]	4	Various	4.8-14.8
SIHON (53)	55	fPB	unknown	4	CD45-CD14	15-60
NIH/NIAID (54)	5	fPB	85-908	144	CD45-CD14	5.5-19.2
GEIL (16)	35	sPB	408	1	Various	27-30
UK NEQAS (31)	77-102	sPB	546-1,335	10	Various	15.0-37.3
WGLSA/ISC (55)	7	fPB, sfPBMC	unknown	49	FACSCount™ single pltf[7]	2.48-8.43
NIH/NIAID (56)	5	fPB	141-611	87	CD45-SSC single pltf[7]	1.8-6.5
SIHON (19)	55	sPB	869	1	CD45-SSC single pltf[7]	8.0-20.0
QASI (57)	23-92	sPB	132->400	9	Various	1.9-72.2
UK NEQAS (58)	296	sPB	140-1,390	108	Various	4.8-56.0
BEQAS (59)	41	fPB	unknown	17	Various	3.7-16.2

Table 2: [1] Literature citations are numbered and listed between brackets; the numbers correspond to those used in the Literature Cited section; [2] Abbreviations: NIH/NIAID, National Institutes of Health / National Institute of Allergy and Infectious Diseases; ACTG, AIDS Clinical Trial Group; CAP-FCS, College of American Pathologists – Flow Cytometry Survey; GIC, Gruppo Italiano di Citometria; NIH/MACS, National Institutes of Health / Multicenter AIDS Cohort Study; CEQUAL, Quality Control Program in Immunophenotyping for Central Europe; SIHON, Stichting Immunofenotypering Hematologische Oncologie Nederland; GEIL, Groupe d'Etude Immunologique des Leucémies; UK-NEQAS, United Kingdom National External Quality Assessment Schemes; WGLSA/ISC, Working Group on Lymphoid Subset Analysis of the Iberian Society of Cytometry; NIAID/DAIDS, National Institute of Allergy and Infectious Diseases / Division of AIDS; QASI, Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS; BEQAS, Belgian national External Quality Assessment Scheme; [3] Abbreviations: f, fresh; s, stabili-zed; sf, stained-fixed; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; [4] Number of CD4+ T cells 106/L; [5] Percentage of CD4+ T cells within leukocyte denominator; [6] Paired heparin and EDTA anticoagulated samples; [7] Abbreviation: pltf, platform; [8] Overall lymphocyte subsets (e.g., CD2+ T cells, CD3+ T cells, CD4+ T cells, CD8+ T cells, CD19+ B cells, and/or NK cells).

CD3+, CD4+ and CD8+ T-cell counts associated with MNC isolation is due to such losses.

Third, sample stabilization showed a bias towards high CD3+, CD4+, CD8+ T-cell counts as well as high NK-cell counts. This result may be explained by some decay of the fresh blood samples during shipment and storage prior to testing. The absence of this bias for B-cell counts would be explained by the observation that loss of CD19 intensity occurs upon stabilization (17,18,63).

Fourth, for gating strategies, the 'CD45-SSC' strategy stood out by a positive bias for CD3+ and CD4+ T-cell counts. Currently, the view is widely held that 'CD45-SSC' gating of lymphocytes is the most robust (64-66). Furthermore, the 'dual-anchor' gating strategy was associated with a negative bias towards NK cells. This method defines a 3-dimensional gate using FSC, SSC and CD45, and has been optimized for single-platform T-cell subset enumeration based on the CD3 positivity of T cells (18). However, a disadvantage of this approach may be the selective loss of CD3 negative lymphocytes, thus underestimation of NK-cell counts, due to differences between CD3+ and CD3- cells in CD45 expression and light scatter characteristics.

Three variables had significant effects on the variability of flow cytometric lymphocyte subset enumeration: (i) platform methodology; (ii) sample stabilization; and (iii) individual laboratories.

First, in single platform assays, the variability of CD4+ and CD8+ T-cell counts was smaller compared to dual-platform assays. This observation confirms previous studies (19,31,56,67,68). In contrast, we observed greater variability in B-cell counts in single-platform assays. The fact that the CD19+ B-cell proportion was very small in most of the distributed samples may explain this result. In other studies (e.g., 13), the CV's of CD19+ B cells was significantly higher then those obtained with CD4+ and CD8+ T cells for both platforms, which is concordant with out data.

Second, for sample stabilization, the variability of CD3+ and CD4+ T-cell counts as well as NK-cell counts was larger when stabilized samples were tested, whilst the variability of B-cell counts was smaller in comparison to non-stabilized samples. In contrast, when using between-laboratory CV as parameter, the variability of CD3+, CD4+ and CD8+ T-cell counts was smaller when stabilized samples were tested as compared to fresh samples (13). In line with our results, a similar comparative study (16) showed a larger variability with stabilized samples. These samples were only a minority of test samples (i.e., 5 of 80) and the participants were not familiar with the (slightly) different light scatter characteristics and fluorescence patterns of stabilized specimens.

Several individual laboratories stood out by a significant higher or lower outcome (i.e., bias) and/or variability relative to the benchmark laboratory. As this result was obtained in a multivariate analysis, such bias and variability must have been due to

other factors than the categorical variables we included in our study. Due to the limitations imposed by the study design, it was not possible to trace the causes of large bias or variability in individual cases.

As clearly indicated by the results of our study, the state of the art methodology for lymphocyte subset enumeration should be a single platform assay with CD45-SSC gating strategy, as proposed by the CDC guidelines (69) and introduced to the participants in a practical workshop (19). At the moment, this methodology is most robust and shows the lowest variability between laboratories. However, participants of the Benelux EQA for flow cytometric lymphocyte enumeration standardized their techniques only partially over time (i.e., with respect to sample preparation and gating strategies, but not with absolute counting techniques). Only when participants are fully committed to standardize their techniques, can significant reductions in between-laboratory variation of lymphocyte subset enumeration be achieved.

8.3.3 Future developments in flow cytometric lymphocyte subset enumeration

One of the most important clinical utilities of flow cytometric lymphocyte subset enumeration is CD4+ T-cell counting for monitoring the disease associated with the human immunodeficiency virus (HIV). With the help of baseline CD4 counts, appropriate timing of starting antiretroviral therapy (ART) and prophylaxis against opportunistic infections is possible (70,71). With the availability of inexpensive ART for resource-restricted areas, attention is also focused on the costs and methodologies of flow cytometric CD4+ T-cell enumeration. Our study underlines that a single platform assay with CD45-SSC gating strategy is favorable. However, in resource restricted areas, the costs (expenses associated with counting beads, utilized in most single platform methodologies) of conventional flow cytometry dedicated to HIV immunophenotyping is prohibitive. At the moment, various studies investigate drastic cost savings and simplifying protocols for flow cytometric CD4+ T-cell enumeration (61,72-74)

With a new generation of bench-top flow cytometers, suspension array technology (SAT) based bead array assays as well as leukocyte immunophenotyping is now available. These hybrid instruments provide an opportunity for the development of a more cost effective multitasking platform to support infectious disease treatment in resource limited countries. For example, a HIV protein bead array combined with cell based T-cell enumeration assay could offer great versatility since various combinations of viral proteins or peptides can be adapted to a variety of situations including HIV surveillance, screening, confirmatory testing, and specific prophylactic vaccine studies (75). This combination of bead and cell based assays on a single hybrid instrument demonstrated the potential utility of a more cost effective multitasking platform.

For more complex flow cytometric immunophenotyping, CD45-SSC based double-anchor approaches are suitable. With the double-anchor approaches, lymphocytes are selected first on the basis of bright CD45 expression and low SSC, followed by the selection of lineage defining or specific markers (e.g.; CD19, CD22, and/or CD79a for B cells CD2, CD3, and/or CD7 for T cells; and CD16, and/or CD56 for NK cells). For classification of hematological malignancies, subtyping within a cell lineage of choice using extensive counterstaining with additional markers is possible.

8.4 EXTERNAL QUALITY ASSESSMENT SCHEMES FOR FLOW CYTOMETRIC APPROACHES

8.4.1 Introduction

During the '90s, clinical cell analysis by flow cytometry has entered the same process of development of instrumental validation, quality control, and EQA as any other clinical laboratory activities. However, compared to other fields of clinical pathology, the progress in establishing a quality assurance process in clinical cell analysis by flow cytometry has been particularly slow. The delicacy of instrumentation, the rapidly evolving hardware and software, the continuous development of new biological targets, monoclonal antibodies, fluorochromes, and most important the brand new area of clinical cytometry complicated the rigorous implementation of such a quality assurance process (76).

In Europe, most EQA schemes were developed from studies organized by local scientific societies often focusing on specific methodological details, in order to obtain technical guidelines on a nationwide basis. In the same way, the aim of the Benelux EQA schemes is to provide feedback to participant laboratories on their overall technical performance. The results of the EQA can be used by the laboratory management to ensure good laboratory practice, and by inspectors in assessing the compliance of a laboratory with good laboratory practice. Although the Benelux EQA schemes provide an advisory service, they do not have a policing role and do not advise whether a particular reagent is superior or not, nor provide reagents. They do provide a cumulative summary of the results and each participant receives detail of its individual laboratory performance and unsatisfactory performance is highlighted. Comparison of performance with other laboratories using the same methodology and an overall comparison of methodologies is also provided.

8.4.2 Evidence based good laboratory practice

The analysis of data from multicenter EQA exercises has resulted in important information with regard to best laboratory practice, such as (i) the use of two HLA-B27 mAb (i.e., FD705 and GS145.2 based HLA-B27 Kit) with non-overlapping cross-reactivity patterns in flow cytometric HLA-B27 screening, (ii) the demonstration of the superiority of single-platform ISHAGE protocol for flow cytometric CD34+ cell enumeration, and (iii) the need of a single platform methodology with, at a minimum, CD45 and SSC based gating strategy for flow cytometric CD4+ T cell enumeration to reduce inconsistencies within and between laboratories. In this way, a number of 'common sense' yet undemonstrated cytometric issues were addressed and then clearly defined by focused EQA studies. When is demonstrated which procedures should be used, the organization of practical workshops in which these protocols are introduced to the participants, is crucial. These workshops, followed by targeted training and feedback in response to reported results, were held on a regular base within the Benelux EQA schemes.

For quantitative flow cytometric approaches, such as CD34+ cell and lymphocyte subset enumeration, our analysis (i.e., robust multivariate regression) of the Benelux EQA exercises clearly shows the effect (or lack of effect) of various methodological variables on the outcome of the assays. Therefore, when a laboratory switches from methodologies, there is a need to document the effect on the reference values, the so called switch study. In common, when normal value ranges were requested utilized during 10 years of Benelux EQA scheme for flow cytometric lymphocyte subset enumeration, only 31% of the participants changed the normal value ranges after a switch in platform methodology, and 16% of the participants changed the normal value ranges after a switch in gating strategy (data not shown). For the laboratories which performed switch studies, 82% reported changes in CD3+ T cell and CD19+ B cell, 91% reported changes in CD4+ and CD8+ T cell, and 73% reported changes in NK cell reference values (data not shown). Regulatory bodies should verify if participants comply with their standards regarding switch studies. In this context it is relevant that the Benelux EQA schemes act as teaching and educational devices.

8.4.3 EQA and laboratory accreditation

As the results are increasingly being used for laboratory accreditation, it is important that EQA schemes themselves meet rigorous quality demands. This implies an impressive degree of organization, documentation, and internal auditing (77,78) of the EQA scheme. Only large and adequately funded institutions can afford such a task and keep the appropriate quality of the sample materials with time. Accredited EQA schemes must take care of a number of items, including (i) documentation of the quality of distributed samples, (ii) the use of validated procedures for evaluating results, (iii) the tracking of poor performers, and (iv) to offer them the required assistance to

achieve or regain an acceptable quality of their work. In this respect, (i) development and validation of sample stabilizing techniques, (ii) accredited reference laboratories were appropriate, and (iii) uniform defined scoring systems, feasible for the underlying assays, are indispensable. For such demands, the development of uniform and statistically appropriate methods which, at the same time, should allow for comparable results between the different laboratories and the monitoring of a laboratory's poor individual performance, is necessary.

In addition, small-scale studies organized by specialized interest groups can focus easily on applications that are continuously posed by the newest developments in clinical cell analysis. When the clinical utility of such applications is shown, this can be implemented in EQA schemes organized in an accredited environment as mentioned above, with as consequence the continuous improvement of laboratory practice through reporting and feedback. This will be the challenge for flow cytometric EQA schemes in the immediate future.

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SUMMARY

Flow cytometry (FCM) uses the principles of hydro-dynamic focussing, light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells. The introduction of the cluster of differentiation (CD) nomenclature in 1980 facilitated the communication of flow cytometric typing results, and consequently FCM rapidly became the method of choice for lymphocyte immunophenotyping in clinical diagnostic laboratories. In the 1990's, flow cytometric CD34+ cell enumeration gradually replaced the culture-based assays to quantify HPC in peripheral blood and bone marrow samples. In the same period, flow cytometry was introduced as a tool for HLA-B27 screening, which was used as a diagnostic criterion for e.g. ankylosing spondylitis (AS). With the increased dependence on FCM for clinical decision-making, various efforts to standardize testing and reduce variability between laboratories have been undertaken. Also, clinical cell analysis by flow cytometry has entered the same process of development of instrumental validation, internal and external quality control (QC), and External Quality Assessment (EQA) as any other laboratory medicine activities.

In this thesis, we analyze the impact of these standardization efforts on clinical cell analysis by FCM with special attention to the external quality assurance programs which currently are in operation for HLA-B27 screening, CD34+ cell enumeration, and lymphocyte subset enumeration.

CHAPTER 1

Chapter one provides a general introduction to the history of FCM and the introduction of FCM in clinical diagnostic laboratory. Special attention with an overview of the literature is given to the clinical background, technical aspects, and external quality assessments regarding HLA-B27 screening, CD34+ cell enumeration, and lymphocyte subset enumeration.

CHAPTER 2

In chapter two, we retrospectively analyzed the results of 7 EQA send-outs to assess the quality of flow cytometric HLA-B27 screening in the Benelux countries. Twenty-eight specimens from blood bank donors, who had been typed for HLA class I antigens, were distributed. Ten samples were HLA-B27^{pos} (all HLA-B*2705) and 18 were HLA-B27^{neg}.

For flow cytometry, the most widely monoclonal antibody (mAb) used was FD705, followed by GS145.2 and ABC-m3. The HLA-B27^{pos} samples were correctly classified as positive by the large majority of participants, the classification of HLA-B27^{neg} samples as negative was less straightforward. Ten samples were correctly classified as such by 97% of the participants, whereas only 64% of the participants correctly classified the remaining eight samples as HLA-B27^{neg}. Inspection of the reactivity patterns of the individual mAb with HLA-B27^{neg} samples revealed that ABC-m3 showed very little cross-reactivity apart from its well-known cross-reactivity with HLA-B7, whereas the cross-reactivity patterns of GS145.2 and FD705 were more extensive. However, the small sample size (n=18) and the distribution of HLA-B antigens other than HLA-B27 did not allow assignment of specificities to these cross-reactions. We showed that standardized interpretation of the combined results of two anti-HLA-B27 mAb reduced the frequency of false-positive conclusions on HLA-B27^{neg} samples.

CHAPTER 3

Due to the small sample size and the distribution of HLA-B antigens other than HLA-B27 in chapter 2, we further analyzed the cross-reactivity patterns of commercially available anti-HLA-B27 mAb (ABC-m3, n = 3; FD705; and GS145.2) from five manufacturers with an extensive cell panel with other HLA-B antigens. Test cells were selected as to express HLA-B antigens with known serologic cross-reactions (HLA-B7, B12, B13, B16, B17, B22, B37, B40, B41, B42, B47, and B48). Cells without B27 cross-reactive antigens (B5, B8, B14, B15, B21, and B35) and cells positive for B27 were included as controls. All mAbs tested showed cross-reactivity, ranging from weak (±) to strong (+), with different antigens and different degrees of intensity. If one mAb had been used for HLA-B27 screening, ABC-m3 would have yielded nine false-positive B27 assignments, FD705 would have yielded seven, and GS145.2 would have yielded two. This problem largely can be avoided by the combined use of two different mAb. The combination of FD705 and GS145.2 yielded the best results, with one false-positive HLA-B27 assignment among the 99 HLA-B27^{-} samples of this highly selected panel.

CHAPTER 4

The findings in chapter 2 and 3 resulted in a proposal for a standard operating procedure for flow cytometric HLA-B27 screening, which is published and nowadays adopted by Current Protocols in Cytometry. Published in affiliation with the International

Society for Analytical Cytology, Current Protocols in Cytometry is a "best practices" collection of peer-reviewed protocols for flow and image cytometry.

CHAPTER 5

In chapter five, we evaluated the suitability of long-term stabilized whole blood specimens as positive or negative procedure controls for flow cytometric HLA-B27 screening, and as test samples in an external quality assessment (EQA) scheme. Peripheral blood samples were obtained from 9 blood bank donors with known HLA-B typing. Commercially available HLA-B27 mAb were tested periodically between 1 week and 12 months and the forward scatter (FSC), sideward scatter (SSC), and fluorescence signals of lymphocytes were compared as a function of time. Furthermore, a pilot send-out with stabilized blood samples of 4 blood bank donors was distributed among the participants to the Benelux EQA scheme for HLA-B27 screening and results were compared with historical EQA data obtained using non-stabilized blood samples from the same donors. Matrix effects were observed, however there were no major effects on FSC and SSC characteristics of lymphocytes. Fluorescence of stabilized samples increased as compared to fresh samples, but the discrimination between HLA-B27 positive and HLA-B27 negative samples remained feasible post stabilization in most cases. In the pilot EQA send-out the results obtained with stabilized samples were less concordant due to variable quality of these samples. In our opinion, long-term stabilized whole blood samples may be useful as true HLA-B27 positive and true HLA-B27 negative control cells for daily and longitudinal quality control of flow cytometric HLA-B27 screening, and may be used for EQA purposes. However, long-term stabilized samples are not suitable for reagent validation purposes. Extensive quality control of stabilized samples is necessary before distribution to the laboratories.

CHAPTER 6

To assess the effect of multiple variables on CD34+ cell enumeration, we analyzed the results of 9 EQA send-outs in the Benelux countries. Therefore, robust multivariate regression, divergent to the statistics performed in most other studies, was used. We studied the effect of methodological aspects of CD34+ cell counting on assay outcome and whether or not this exercise was effective in reducing between-laboratory variation. Five variables were associated with significant bias of absolute CD34+ cell counts: (i) laboratory, (ii) gating strategy; (iii) CD34 mAb fluorochrome; (iv) type of flow cytometer, and (v) method of sample preparation. In addition, laboratory and

platform methodology (i.e., single vs. dual) contributed significantly to the variability of this assay. Our approach, i.e., EQA with targeted training and feedback in response to reported results, has been successful in reducing the variability of CD34+ cell enumeration between participants.

CHAPTER 7

To assess the effect of multiple variables on CD3+ T-cell, CD4+ T-cell, CD8+ T-cell, NK-cell, and CD19+ B-cell enumeration, we analyzed the results of 10 EQA send-outs in the Benelux countries. Again, robust multivariate regression was used. We studied the effects of the methods used on assay outcome, and whether or not this EQA exercise was effective in reducing between-laboratory variation. Five variables were associated with significant bias of absolute lymphocyte subset counts: (i) platform methodology, (ii) sample preparation technique, (iii) gating strategies, (iv) sample stabilisation, and v) laboratory. Platform methodology, sample stabilization, and laboratory also affected assay variability. With time, assay variability tended to decline; this trend was significant for B-cell counts only. Participants tended to standardize their techniques mainly with respect to sample preparation and gating strategies, but less with absolute counting techniques. Failure to fully standardize protocols may have led to only modest reductions in variability of results between laboratories.

CHAPTER 8

Chapter 8 is the general discussion of this thesis. The educational aspects of EQA's regarding HLA-B27 screening, CD34+ cell enumeration, and lymphocyte subset enumeration are described. In addition, trends toward improvement as well as future developments for the above mentioned flow cytometric applications are described. Finally, the role of EQA schemes for flow cytometric in terms of evidence based good laboratory practice, laboratory accreditation, Europe, and new types of assays is discussed.

SAMENVATTING

Flowcytometrie (FCM) maakt gebruik van de principes van hydrodynamische focussering, licht verstrooiing en fluorescentie om verschillende eigenschappen van deeltjes en cellen te meten. De introductie van de CD-nomenclatuur in 1980 maakt het vervolgens mogelijk eenduidig over deze eigenschappen te communiceren, waarna FCM al snel dé techniek wordt voor lymfocyt fenotypering binnen klinisch diagnostische laboratoria. In de 90-er jaren vervangt de flowcytometrische CD34 bepaling geleidelijk de dan gebruikelijke kweektechnieken voor het kwantificeren van CD34+ cellen in perifeer bloed en beenmerg. Tegelijkertijd komt de flowcytometrische HLA-B27 bepaling in zwang, gebruikt bij de diagnostiek van bijvoorbeeld de ziekte van Bechterew. Het toenemend gebruik van flowcytometrie voor diagnostische doeleinden maakt standaardisatie van de gebruikte technieken en vergelijkbare resultaten tussen laboratoria onderling noodzakelijk. Ook spelen validaties, interne en externe kwaliteitscontroles en ringonderzoeken voor flowcytometrische technieken een steeds belangrijkere rol.

Dit proefschrift beschrijft de impact van standaardisatie op de kwaliteit van flowcytometrische technieken met speciale aandacht voor de rol van ringonderzoeken voor flowcytometrische HLA-B27 screening, CD34 bepaling en lymfocyt subset bepaling in deze.

HOOFDSTUK 1

In hoofdstuk 1 wordt een algemene introductie gegeven over de historie van FCM en de intrede van FCM in klinisch diagnostische laboratoria. Speciale aandacht, met een overzicht van de literatuur, wordt besteed aan klinische achtergronden, technische aspecten en bestaande ringonderzoeken m.b.t. flowcytometrische HLA-B27 screening, CD34 bepaling en lymfocyt subset bepaling.

HOOFDSTUK 2

In hoofdstuk 2 wordt een retrospectieve analyse van de resultaten van 7 ringonderzoeken m.b.t. flowcytometrische HLA-B27 screening binnen de Benelux weergeven. Achtentwintig bloedmonsters van bloedbankdonors met een bekende HLA-klasse I typering werden verzonden. Tien monsters waren HLA-B27 positief (allen HLA-B*2705) en 18 monsters waren HLA-B27 negatief. Het monoklonaal FD705 werd het meest gebruikt, gevolgd door de monoklonalen GS145.2 en ABC-m3. De overgrote meerderheid van

de deelnemers typeert eenduidig de HLA-B27 positieve monsters. De typering van de HLA-B27 negatieve monsters was minder eenduidig. Tien monsters werden eenduidig getypeerd als HLA-B27 negatief door 97% van de deelnemers, de overige 8 monsters werden echter slechts door 64% van de deelnemers eenduidig getypeerd als HLA-B27 negatief. Analyse van de reactiepatronen van de monoklonalen leert dat ABC-m3 weinig kruisreactiviteit laat zien naast de reeds bekende kruisreactiviteit met HLA-B7. De kruisreactiviteit van de monoklonalen GS145.2 en FD705 blijkt groter. Echter, het laag aantal waarnemingen (n=18) en de verdeling van HLA-B antigenen anders dan HLA-B27 laten geen nauwkeurig analyse van reactiepatronen van de monoklonalen toe. Wel wordt aangetoond dat een gestandaardiseerde interpretatie van gecombineerde resultaten van twee anti-HLA-B27 monoklonalen leidt tot minder foutpositieve reacties.

HOOFDSTUK 3

Door het laag aantal waarnemingen en slechte diversiteit van HLA-B antigenen anders dan HLA-B27 in hoofdstuk 2 is een verdere analyse verricht van de kruisreactiviteit van commercieel verkrijgbare anti-HLA-B27 monoklonalen (ABC-m3, n=3; FD705 en GS145.2) van 5 leveranciers tegen een uitgebreid panel van HLA-B antigenen. Cellen zijn geselecteerd op expressie van HLA-B antigenen met bekende serologische kruisreacties (HLA-B7, B12, B13, B16, B17, B22, B37, B40, B41, B42, B47 en B48). Cellen zonder B27 kruisreagerende antigenen (B5, B8, B14, B15, B21 en B35) en cellen positief voor B27 zijn als controles toegevoegd. Al de geteste monoklonalen vertonen kruisreactiviteit met verschillende antigenen en mate van intensiteit. Wanneer 1 monoklonaal zou worden gebruikt, zou ABC-m3 negen foutpositieve resultaten, FD705 zeven foutpositieve resultaten en GS145.2 twee foutpositieve resultaten hebben opgeleverd. Dit probleem wordt grotendeels voorkomen door een combinatie van twee monoklonalen te gebruiken. De combinatie van FD704 met GS145.2 behaald hierbij de beste resultaten, met één foutpositief resultaat binnen de 99 HLA-B27 negatieve monsters van het geselecteerde panel.

HOOFDSTUK 4

De bevindingen uit hoofdstuk 2 en 3 hebben geresulteerd in een voorstel voor een ‘Standard Operating Procedure’ voor flowcytometrische HLA-B27 screening, welke is gepubliceerd en opgenomen in Current Protocols in Cytometry. Current Protocols in

Cytometry is een verzameling van 'best practices' protocollen, peer-reviewed, voor flow- en beeldcytometrie.

HOOFDSTUK 5

In hoofdstuk 5 wordt een studie beschreven waarin de bruikbaarheid van gestabiliseerde bloedmonsters als positieve of negatieve controles en als monsters voor EQA doeleinden voor flowcytometrische HLA-B27 screening wordt onderzocht. Gedurende 1 week tot 12 maanden worden periodiek gestabiliseerde bloedmonsters van 9 bloedbankdonors met bekende HLA-typeringen getest met commercieel verkrijgbare HLA-B27 monoklonalen. In de tijd worden voorwaartse lichtverstrooiing (FSC), zijwaartse lichtverstrooiing en fluorescentie signalen van lymfocyten vergeleken. Tevens is een EQA rondzending georganiseerd waarin vier gestabiliseerde bloedmonsters zijn verzonden die reeds eerder vers zijn verzonden, waarna de resultaten met elkaar zijn vergeleken. Matrixeffecten zijn waarneembaar, maar hebben geen substantieel effect op de FSC en SSC eigenschappen van lymfocyten. De achtergrondfluorescentie neemt toe vergeleken met verse monsters, maar het onderscheid tussen HLA-B27 positieve en HLA-B27 negatieve monsters blijft in veel gevallen mogelijk. De resultaten behaald in de EQA rondzending zijn slechter voor gestabiliseerde monsters dan voor verse monsters, met name door een variërende kwaliteit van deze monsters. Gestabiliseerde monsters zijn mogelijk bruikbaar als positieve en negatieve controles en als monsters voor EQA doeleinden, maar zijn niet geschikt voor validatiedoeleinden. Een intensieve kwaliteitscontrole van de gestabiliseerde monsters is echter noodzakelijk voordat deze binnen de laboratoria kunnen worden toegepast.

HOOFDSTUK 6

Om het effect van diverse variabelen binnen flowcytometrische CD34 bepalingen op het CD34 getal vast te stellen zijn de resultaten van 9 EQA rondzendingen geanalyseerd. Hierbij is, in afwijking van eerdere studies, gebruik gemaakt van robuuste multivariaat regressie analyse. Het effect van methodologische aspecten van flowcytometrische CD34 bepalingen op het CD34 getal en een mogelijk positief effect van de EQA op de interlaboratorium variabiliteit zijn onderzocht. Vijf variabelen laten systematische verschillen zien, te weten laboratorium, gating strategie, fluorochroom CD34 monoklonaal, flowcytometer en monsterpreparatie. Verder hebben de variabelen laboratorium en platform techniek invloed op de variabiliteit van de flowcytometrische CD34 bepaling. De aanpak van EQA gecombineerd met specifieke training van de

deelnemers en het bespreken van de resultaten in plenaire sessies blijkt effectief voor de reductie van de interlaboratorium variabiliteit.

HOOFDSTUK 7

Om het effect van diverse variabelen binnen flowcytometrische lymfocyt subset bepalingen op het absoluut aantal T-, B- en NK-cellen vast te stellen zijn de resultaten van 10 EQA rondzendingen geanalyseerd. Hierbij is opnieuw gebruik gemaakt van robuuste multivariaat regressie analyse. Het effect van diverse methodieken op systematische verschillen en variabiliteit en een mogelijk positief effect van de EQA op de interlaboratorium variabiliteit zijn onderzocht. Vijf variabelen laten systematische verschillen zien, te weten platform techniek, monsterpreparatie, gating strategie, monsterstabilisatie en laboratorium. De variabelen platform techniek, monsterstabilisatie en laboratorium hebben eveneens invloed op de variabiliteit van de flowcytometrische lymfocyt subset bepaling. Gedurende de tijd lijkt de variabiliteit van de lymfocyt subset bepaling af te nemen, dit effect is echter alleen significant voor het absoluut aantal B-cellen. Deelnemers bereiken hoofdzakelijk een consensus over monsterpreparatie en gating strategie, echter niet over platformtechniek. Het ontbreken van een volledige consensus over een gestandaardiseerde flowcytometrische lymfocyten subset bepaling kan de oorzaak zijn van de slechts minimale reductie van de interlaboratorium variabiliteit.

HOOFDSTUK 8

Hoofdstuk 8 is de algemene discussie van dit proefschrift. De educatieve aspecten van EQA's voor flowcytometrische HLA-B27 screening, CD34 bepaling en lymfocyt subset bepaling, als ook trends tot verbetering en toekomstige ontwikkelingen worden beschreven. De rol van EQA rondzendingen in het kader van good laboratory practice, laboratorium accreditatie, Europese samenwerking en nieuwe technische ontwikkelingen wordt eveneens belicht.

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LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin
aGVHD	acute graft versus hose disease
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	allophycocyanin
AS	ankylosing spondylitis
ASHI	American Society for Histocompatibility and Immunogenetics
BCSH	British Committee for Standards in Haematology
BM	bone marrow
BVCA/ABCA	Belgian Association for Analytical Cytometry
CAP	College of American Pathologists
CD	cluster of differentiation
CDC	Centers for Disease Control
CDC	complement dependent cytotoxicity
CFC	colony-forming-cell
cGVHD	chronic graft versus hose disease
CLSI	Clinical and Laboratory Standards Institute
CREG	cross-reacting group
CTN	Canadian HIV Trials Network
CV	coefficient of variation
DIAIDS	Division of AIDS
EBMT	European Group for Blood and Marrow Transplantation
EFI	European Federation for Immunogenetics
EWGCCA	European Working Group on Clinical Cell Analysis
FCM	flow cytometry
FITC	fluorescein isothiocyanate
FS	forward light scatter
FSC	forward light scatter
FTR	further typing required
G-CSF	granulocyte colony stimulating factor
GEIL	Groupe d'Etude Immunologique des Leucémies
GM-CSF	granulocyte macrophage colony stimulating factor
GVL	graft-versus-leukaemia
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPC	haematopoietic stem and progenitor cells

HSC	haematopoietic stem and progenitor cells
ISCT	International Society for Cell Therapy
ISHAGE	International Society of Hematotherapy and Graft Engineering
JACIE	Joint Accreditation Committee-ISCT & EBMT
PCR	polymerase chain reaction
mAb	monoclonal antibody
MAb	monoclonal antibody
MESF	molecules of equivalent soluble fluorochrome
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MoAb	monoclonal antibody
MRD	minimal residual diseases
NAD	nucleic acid dye
NIAID	National Institute for Allergic and Infectious Diseases
NIAIDS	National Institute for Allergic and Infectious Diseases
NVC	Nederlandse Verening voor Cytometrie
NXR	non cross-reactive
OSGE	O-sialoglycoprotein endopeptidase
PBSC	peripheral blood stem and progenitor cells
PE	phycoerythrin
QMP-LS	Canadian Quality Management Programme - Lab Services
SCT	stem cell transplantation
SIHON	Dutch Foundation for Immunophenotyping in Hemato-Oncology
SKML	Dutch Foundation for Quality Control of Medical Laboratory Diagnosis
SOP	Standard Operation Procedure
SpA	(related) spondyloarthropathy
SS	sideward light scatter
SSC	sideward light scatter
SSO	sequence specific oligonucleotides
SSP	sequence specific primers
TdT	like terminal deoxynucleotidyl transferase
TRM	transplant-related mortality
UCB	umbilical cord blood
VCN	Vibrio cholera neuraminidase
WHO	World Health Organization

CURRICULUM VITAE

Wilfridus Henricus Bernardus Maria Levering was born on May 2nd 1965 in Roosendaal. He attended primary school at the Turfvaart, and secondary school at the Athenaeum of the Gertrudislyceum in Roosendaal, where he graduated in 1983. In the same year he started to study as technologist in clinical chemistry at the Dr. Struycken-instituut in Etten-Leur, where he graduated in 1987. In 1989, after a period at the Foundation for Medical Laboratories (dr. R. Scholtis) in Breda, he started to work at the departments Immunohematology and Immunocytology (dr. R.L.H. Bolhuis) of the Erasmus MC – Daniel den Hoed in Rotterdam. In the early 1990's, he was introduced to flow cytometry and became an active member of the SIHON Flow Cytometry Working Party. In 1993, he started to work at the Laboratory for Histocompatibility and Immunogenetics (dr. K. Sintnicolaas) of the Blood Bank Rotterdam. In 1995, under the auspices of the SIHON, SKML, and BVCA/ABCA, EQA's for flow cytometric HLA-B27 screening, CD34+ cell enumeration and lymphocyte subset enumeration were started with the help of three reference laboratories: the department of Immunology (prof. dr. H. Hooijkaas) and the department of Medical and Tumor Immunology (dr. J.W. Gratama) of the Erasmus MC, and the Laboratory for Histocompatibility and Immunogenetics (W. Levering) of the Blood Bank Rotterdam. In 1995, he started to work as general supervisor of the LHI. Since September 1st 2003, he started a training as technical director of the LHI of Sanquin Blood Bank South West Region. As part of the training, the research as described in this thesis was performed. At the moment he is active as technical director of the LHI of Sanquin Blood Bank South West Region, member of the Flow Cytometry Working Party of the Dutch Association for Cytometry and advisor of the Clinical and Laboratory Standards Institute.

He lives together with his wife Karin and daughters Sanne and Femke and likes to spend his time with motorcycling, music, and photography.

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